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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> GENE SCREENING METHODS AND RELATED ASSAYS  <b>(57) Abstract</b>  The present invention relates to cell death genes, which are genes required for programmed cell death; mutant organisms, in which ectopic programmed cell death occurs in embryos to a greater than normal extent and agents which alter the ability to modulate programmed cell death of cells. A mutant form of <i>Dakt1</i> , <i>Dakt1<sup>g</sup></i> in a <i>Drosophila melanogaster</i> has been identified. <i>Dakt1</i> and its mammalian homolog, PKB are known to protect cells from undergoing apoptosis. A genetic screen for identifying genes related to programmed cell death by their ability to interact genetically with the mutant form of <i>Dakt1</i> is described.		

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## GENE SCREENING METHODS AND RELATED ASSAYS

### CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. provisional application Ser. No.60/083,077, filed April 27, 1998, the contents of which is hereby incorporated in its entirety by reference.

5

### FIELD OF THE INVENTION

The present invention relates generally to the fields of genetics and molecular biology and, in particular, to genetic control of programmed cell death (PCD), or apoptosis in insect and human cells.

### BACKGROUND OF THE INVENTION

The decision between survival and death is an important aspect of cellular regulation during development and malignancy. Central to this regulation is the process of programmed cell death (PCD) or apoptosis, which is conserved in multi-cellular organisms (Jacobson MD, *et al. Cell* 1997, 88:347-354.) While there has been rapid progress in elucidation of the cellular apoptotic processes such as the caspases, the mechanisms by which this critical process is regulated are not clearly understood (Harrington *et.al. EMBO J.* 1994, 13:3286-3295.) A variety of signalling cascades has been implicated in modulation of PCD including the phosphatidylinositol 3' kinase(PI3'K) pathway. Activation of PI3'K, a lipid kinase, protects against PCD, whereas it's inhibition enhances PCD (Yao R, *et al. Science* 1995, 267: 2003-2006. Kauffmann-Zeh *et al. Nature* 1997, 385:544-548. Kennedy *et al. Genes Dev.* 1997, 11:701-713. Kulik *et al. Mol. Cell. Biol.* 1997 17:1595-1606. Dudek *et al. Science* 1997, 275:661-665. Marte *et al. TIBS* 1997, 22:355-358.) Recently, the protective effects of PI3'K in mammalian cells have been linked to its activation of the protein kinase termed PKB or AKT (Bellacosa *et al. Science* 1991, 254, 274-277). The PKB/AKT gene was identified as the human homologue of a retroviral oncogene named v-Akt, which was isolated from the acute transforming retrovirus, AKT8 (Bellacosa *et al. Science* 1991, 254, 274-277). PKB has been shown to be expressed in brain, thymus, heart and lung. It has also been reported that PKB is overexpressed in some mammalian pancreatic, prostate, ovarian and breast cancers as well as glioblastomas and leukaemia. It is known that defects in the insulin signalling pathway are associated with mutations in PKB. Such diseases may include hyperglycemia, particularly non-insulin dependent diabetes mellitus (NIDDM); by related disorders, which may include obesity, hypertension, hypercholesterolemia, hypertriglyceridemia, etc.; by proliferation disorders, e.g., cancer, restenosis, rheumatoid arthritis; or by loss of bone density, e.g. osteoporosis. PKB has also been implicated in angiogenesis. Much work has focused on the signalling pathway for the activation and function of PKB/AKT in mammals, and despite intense research surrounding the downstream actions of this enzyme, few *in vivo* phosphorylation targets have been identified. While the activation of PKB/AKT in mammals has been linked to protection against PCD, a definitive role for this kinase in PCD has yet to be established. It is also possible that PKB/AKT has other as yet unknown physiological roles not related to PCD.

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The use of knockout mice is a powerful tool for finding information about the function of a gene *in vivo*. In this method a selected gene is inactivated while the remaining genome is unaffected. There are several disadvantages to generating knockout mice. Besides being time consuming and expensive, mouse genetic analysis is slow and complicated. Also, the strategy for generating knockout mice will in many cases delete a gene critical for early embryonic development. In such cases the pups carrying the knocked out gene will die *in utero*. In the particular case of PKB/AKT, the application of knockout strategy is further hampered by the fact that PKB/AKT already exist as three isoforms encoded by three different genes termed PKB $\alpha$ /AKT1, PKB $\beta$ /AKT2 and PKB $\gamma$ /AKT3. The proteins encoded by these genes have high homology with each other.

Because of the difficulties of studying functions of genes in mammals, many researchers have focused their efforts on model systems that are genetically more amenable to manipulation. *Drosophila* genetic studies have been a powerful tool in dissecting signalling cascades and regulatory pathways. Recombinant DNA experiments with *Drosophila* have assisted the clarification of principles and ideas established by decades of classical genetic and embryological studies. There is evidence suggesting that the physiology of PCD is quite highly conserved between mammals and arthropods. The morphological changes associated with programmed cell death are similar in both vertebrates and invertebrates (Kerr, *et al. Br. J. Cancer*, 26: 239-257). A number of viral proteins that suppress PCD in their hosts e.g., p35 and crmA, exhibit potent anti-apoptotic activity in a range of heterologous species (Hay *et al. Development* 120: 2121-2129; Pronk, *et al. Science* 271: 808-810). A number of functional homologues of mammalian PCD factors have been identified in *Drosophila*. For example, counterparts to the caspases are represented as are the inhibitor of apoptosis (iap) proteins (Harrington *et al. EMBO J.* 1994, 13: 3286-3295; Sanson *et al. Nature* 1996, 383: 627-633; Heitzler *et al. Genetics* 1996, 143: 1271-1286; Chou *et al. Genetics* 1996, 144: 1673-1679). A *Drosophila* homologue of PKB/AKT (*Dakt1*) has also been biochemically characterized however corresponding mutants have not been identified (Franc *et al. Immunity* 1996, 4:431-443; Grether *et al. Genes Dev.* 1995, 9: 1694-1708). The *Dakt1* gene spans approximately 5.6Kb of DNA and maps to cytological position 89B on the right arm of the third chromosome of *Drosophila melanogaster*. Like its mammalian homologue, *Dakt1* contains an N-terminal PH domain, a central catalytic kinase domain and the two activation sites at Thr 342 and Ser 505. *Dakt1* has 75% homology to mammalian PKB at the amino acid level and 64% identity at the DNA level. Specifically, the N-terminal PH domain is 71% homologous and the catalytic domain shows 86% homology with conserved motifs for ATP binding and for defining the Ser/Thr specificity of the kinase activity. Both transcription and the intrinsic kinase activity are differentially regulated throughout embryogenesis, larval development, pupariation and between the sexes in adults.

There are clinical implications to understanding the biology of PCD. In normal development of a multicellular organism, many more cells are produced than are needed in the mature or adult tissue. Therefore, the process of cell birth, mitosis, must be counterbalanced by a process for reducing cell number. There is a wide range of diseases in which a disruption of the normal cell death pathway may play a role. In nervous system disorders such as stroke and head trauma, neurons die due to external death triggers such as lack of oxygen. Recent studies suggest that at least a portion of this neuronal death may occur by PCD. In other neuronal diseases, such as

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Alzheimer's disease, and amyotrophic lateral sclerosis, neurons die as part of a degenerative process whose triggers have not yet been identified but which could involve PCD. By contrast, in disorders such as cancer and hyperimmune diseases, cells live where they would normally die. This results in tumors (cancer) or an overactive immune system (autoimmune diseases). Thus, the ability to intervene pharmacologically in the cell death pathway provides the possibility of a new set of therapeutic agents useful in a wide range of human disease. Despite its importance in animal development, a great deal remains unknown about the genetic and molecular mechanisms involved in PCD.

There is thus a need for methods of identifying genes and their products controlling PCD in cells that have a disrupted PCD function by restoration of PCD genes. Results obtained from the study of *Drosophila* can produce insight into other species, including mammalian and even human cells.

#### SUMMARY OF THE INVENTION

One aspect of the present invention is a method of determining whether a mutation capable of enhancing programmed cell death is present in a *Drosophila melanogaster* fly.

The method includes providing a mutant first fly having a recessive first mutation, the presence of which mutation is known to induce programmed cell death in an embryo of a fly homozygous for the mutation. A second mutant fly is provided. This second fly has a mutation which and the method is applied to determine whether such a mutation is capable of enhancing programmed cell death. The first and second flies are crossed with each other. It is then determined whether there are embryos obtained from the crossing of the flies which have undergone ectopic programmed cell death. If it is determined that there are embryos that have undergone ectopic cell death, this indicates that a mutation capable of enhancing programmed cell death is present in the second mutated fly.

According to a particular method, described in detail below, the determination of whether embryos have undergone ectopic programmed cell death includes determining whether at least about 5 percent of the total number of embryos formed have undergone ectopic cell death. In making this determination it must be taken into account that some embryos develop and go on to hatch. In any case, the higher the percentage of embryos that are found to undergo ectopic cell death, the greater the certainty that the second mutant fly has a mutation capable of enhancing programmed cell death. The observed percentage can be 6 percent, 7 percent, or greater. The total observable percentage would be 25 percent, based on the statistics of genetic crossings, but this maximum would rarely, if ever, be observed.

In an illustrated embodiment, the first fly used in the method has been characterized to the extent that it is known to have a mutation in its *DAK1* gene and the mutation is known to induce programmed cell death in an embryo of a fly homozygous for the mutation. The mutation has been observed to result in loss of the kinase activity of the protein encoded by the gene, the "gene product". In particular, the mutation results in a phenylalanine

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to isoleucine substitution at position 327 of the protein. In terms of the gene itself, this mutation has been determined is given below.

In another illustrated embodiment, the first fly is known to have at least one mutation in a DPP2A gene known to induce programmed cell death.

5 DPP2A is known to encode a protein having phosphatase activity, so it is thought that the gene contains a mutation which results in loss of phosphatase activity of the protein encoded thereby.

The second fly, the fly having a mutation for which it is to be determined whether a gene mutation capable of enhancing programmed cell death is present, can be obtained by conventional methods. Particularly, a mutant fly can be obtained by exposing a wild-type fly to ionizing radiation, by chemical mutagenesis of a wild-type fly, or by  
10 inserting P-elements in the genome of a wild-type fly.

In a method of the invention, illustrated in detail below, embryos obtained from the crossing of the flies are initially visually examined (under a microscope). Dead embryos displaying loss of cuticle are selected for determination of whether they have undergone ectopic programmed cell death. Embryos displaying such cuticle loss are treated to degrade or remove the chorion of the embryos. This permits staining of embryonic cells interior  
15 of the chorion for visual determination of the presence of ectopic programmed cell death. The treated embryos are thus treated, as with a dye, and they are visually inspected to determine whether they have undergone programmed cell death.

Particular embryonic treatments include terminal deoxy-transferase mediated hapten-labeled-dUTP nick end labeling (TUNEL) or with acridine orange staining.

20 According to the method, once a mutant fly is determined to have a mutation capable of enhancing programmed cell death, the chromosomal location of mutations in the fly can be determined. Once the location of a mutation is determined, the nucleotide sequence of the mutation can be determined. Sequence information obtained from the mutant can be compared with wild type sequences, as possibly contained in a sequence database, to determine wild type sequence(s) corresponding to the mutant sequence. From this, potentially counterpart  
25 sequences could be located. If a function is known of a protein encoded by a wild type sequence potentially counterpart to that determined for the mutant, the a protein based on the mutant sequence could be created and tested for activity corresponding to that of the potential counterpart. Also, the wild type fly sequence on which the mutant sequence is based, could be determined.

In another broad aspect, the invention is a method of determining whether a mutation capable of disrupting  
30 development is present in a *Drosophila melanogaster* fly. The method includes:

- (a) providing a first mutant fly having a recessive first mutation, the presence of which mutation is known to induce programmed cell death in an embryo of a fly homozygous for the mutation;

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- (b) providing a second mutant fly for determination;
- (c) crossing the flies in (a) and (b);
- (d) examining dead embryos obtained in step (c) to determine whether dead embryos of a phenotype different from either the first or second mutant flies are present;
- 5 (e) wherein, if a said different phenotype is observed then a mutation capable of disrupting development is present in the second mutant fly.

Disrupted, or abnormal, development is evidenced in an embryo by the presence of embryos which fail to experience normal cell fates. In a particular embodiment, illustrated below, step (d) includes visually examining embryos obtained in step (c) to determine whether trachea are missing.

- 10 In another broad aspect, the invention is a method of determining whether a mutation capable of disrupting development is present in a *Drosophila melanogaster* fly, the method comprising:

- (a) providing a first mutant fly having a recessive first mutation, the presence of which mutation is known to disrupt development in an embryo of a fly homozygous for the mutation;
- (b) providing a second mutant fly for determination;
- 15 (c) crossing the flies in (a) and (b);
- (d) examining dead embryos obtained in step (c) to determine whether dead embryos of a phenotype different from either the first or second mutant flies are present;
- (e) wherein, if a said different phenotype is present then a mutation capable of disrupting development is present in the second mutant fly.

- 20 In a particular embodiment, illustrated below, the first fly is known to have at least one mutation in a *trachealess* gene known to disrupt development in an embryo of a fly homozygous for the mutation.

- It is possible, for convenience, to introduce a marker into the chromosome of the mutant second fly at a location suitable to permit identification of a mutation introduced into the second fly, and wherein the method further comprises the step of identifying the mutation. This permits identifying the mutation by sequencing the chromosome of the fly in the vicinity of said marker and comparing the sequence obtained with a wild type sequence to determine the wild type sequence corresponding to the mutant sequence.
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In another aspect, the invention is a method of inducing programmed cell death in a cell by inhibiting the kinase activity of *DAkt1* protein. In particular, the method can include administering a nucleic acid molecule sufficiently complementary such as to inhibit translation of *DAkt1* mRNA present in a cell.

5 In another aspect, the invention is a method of inducing programmed cell death in a cell by inhibiting the phosphatase activity of a *PP2A* protein. In particular, the method can include administering a nucleic acid molecule sufficiently complementary such as to inhibit translation of *PP2A* mRNA present in a cell.

In another broad aspect, the invention is an assay for determining whether a substance is potentially suitable for usage as an active ingredient in inducing programmed cell death or in inhibiting cell death, which assay comprises the following steps:

10 exposing *DAkt1* having kinase activity to the substance;

quantitatively assaying the effect of the substance on the kinase activity; and

determining based on the results of said assay the potential suitability of the substance as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death.

15 Particularly, a said substance is potentially suitable as an active ingredient in inducing programmed cell death if determined to quantitatively inhibit the kinase activity.

Alternatively, a said substance is potentially suitable as an active ingredient in inhibiting programmed cell death if determined to quantitatively enhance the kinase activity.

20

The assay can further include:

exposing embryos of *Drosophila melanogaster* fly to the substance;

quantitatively assaying the effect of the substance on ectopic cell death in the embryos; and

25 determining based on the results of said assay the potential suitability of the substance as an active ingredient in inducing programmed cell death or inhibiting programmed cell death.

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In another aspect, the invention is an assay for determining whether a substance is potentially suitable for usage as an active ingredient in inducing programmed cell death or inhibiting programmed cell death, which assay comprises the following steps:

- exposing PP2A having phosphatase activity to the substance;
- 5 quantitatively assaying the effect of the substance on the phosphatase activity; and
- determining based on the results of said assay the potential suitability of the substance as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death.

Particularly, such a substance is potentially suitable as an active ingredient in inducing programmed cell death if determined to quantitatively inhibit the phosphatase activity.

Particularly, such a substance is potentially suitable as an active ingredient in inhibiting programmed cell death if determined to quantitatively enhance the phosphatase activity.

15 Such an assay can also include:

- exposing embryos of *Drosophila melanogaster* fly to the substance;
- quantitatively assaying the effect of the substance on the ectopic cell death in the embryos; and
- determining based on the results of said assay the potential suitability of the substance as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death.

20

Another assay of the invention is for determining whether a substance is potentially suitable for usage as an active ingredient in arresting development of an organism, wherein the assay includes the following steps:

- exposing a *trachealess* protein capable of initiating transcription to the substance;
- quantitatively assaying the effect of the substance on the ability of the protein to initiate transcription; and
- 25 determining based on the results of said assay the potential suitability of the substance as an active ingredient in arresting development of an organism.

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The assay can further include the following steps:

exposing embryos of *Drosophila melanogaster* fly to the substance;

quantitatively assaying the effect of the substance on development of the embryos; and

5 determining based on the results of said assay the potential suitability of the substance as an active ingredient in arresting development of an organism.

Assaying the effect of the substance on development of the embryos can include determining the effect of the substance on tracheal development.

10

The invention includes a method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for disrupted development, the method comprising the steps of:

providing a sample of genetic material of the subject;

15 determining whether the genetic material includes a nucleotide sequence encoding a mutant *trachealess* protein incapable of initiating transcription, wherein

the presence of said nucleotide sequence indicates the presence of a potential to confer on its offspring a predisposition for abnormal development.

In a particular embodiment of this aspect of the invention, the method also includes:

20 determining whether the genetic material includes a nucleotide sequence encoding a *trachealess* protein capable of initiating transcription.

In a particular aspect, the *trachealess* protein capable of initiating transcription includes the amino sequence identified as SEQ ID NO:1.

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In another aspect, the invention is a method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for abnormal development, the method comprising the steps of:

providing a sample of genetic material of the subject;

determining whether the genetic material includes a nucleotide sequence encoding a mutant PP2A protein lacking phosphatase activity, wherein

the presence of said nucleotide sequence indicates said potential predisposition.

In another aspect, the invention is a method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for abnormal development, the method comprising the steps of:

providing a sample of genetic material of the subject;

determining whether the genetic material includes a nucleotide sequence encoding a mutant Dakt1/PKB protein having kinase activity lower than the activity of wild type dakT/PKB, wherein

the presence of said nucleotide sequence indicates said potential predisposition.

This invention includes a method of inducing programmed cell death in a cell, the method comprising inhibiting the activity of PP2A in the cell. Inhibiting the activity of PP2A in the cell can include administering to the cell an effective amount of an antisense nucleic acid molecule sufficiently complementary to an mRNA encoding PP2A to bind thereto so as reduce translation thereof. Inhibiting the activity of PP2A in the cell can include administering to the cell an effective amount of a phosphatase inhibitor.

The inhibitor can be selected from the group consisting of microcystin LR and caliculin.

In another important aspect, the invention is an assay for determining whether a substance is potentially suitable for usage as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death.

which assay comprises the following steps:

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providing a cell in which a reporter gene is operably linked to a promoter/enhancer which is acted upon by *trachealess*, wherein the reporter gene is one which, in nature, is not operably linked to the sequence promoter/enhancer;

exposing the cell to the substance;

5 quantitatively assaying the effect of the substance on expression of the reporter gene; and

determining based on the results of said assay the potential suitability of the substance as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death.

The cell is preferably a cell of a *Drosophila melanogaster* fly.

10

The promoter/enhancer, in a particular embodiment, includes the nucleotide sequence identified as SEQ ID NO:3.

15 In particular embodiments, the determination step includes comparing the effect of the substance on the expression of the reporter gene with the expression of the reporter gene in the absence of the substance.

The substance being assayed can be a kinase inhibitor or a phosphatase inhibitor.

20 According to a certain aspect of this assay of the invention, a said substance is potentially suitable for usage as an active ingredient in inducing programmed cell death if determined to quantitatively inhibit expression of the reporter gene. Alternatively, a said substance is potentially suitable for usage as an active ingredient in inhibiting programmed cell death if determined to quantitatively enhance expression of the reporter gene.

25 In another aspect, the invention is an assay for determining whether a substance is potentially suitable for usage as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death, which assay comprises the following steps:

exposing a cell to the substance:

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quantitatively assaying the effect of the substance on expression of the *trachealess* gene; and

determining based on the results of said assay the potential suitability of the substance as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death.

5        Such an assay can include determining the amount of RNA encoding *trachealess* produced by the cell in the presence of the substance and comparing the amount with the amount of RNA encoding *trachealess* produced by the cell in the absence of the substance.

10        Determining the amount of RNA encoding *trachealess* can include amplifying the RNA in the presence of primers complementary thereto.

A said substance is potentially suitable as an active ingredient in inducing programmed cell death if determined to quantitatively inhibit said expression. A said substance is potentially suitable as an active ingredient in inhibiting programmed cell death if determined to quantitatively enhance said expression.

15

In this specification, percent homology between amino acid sequences is determined using GCG software using the "best fit" command.

#### BRIEF DESCRIPTION OF THE FIGURES

20    **FIG. 1**     Schematic representation of the genetic screen performed to identify mutations that interact with *Dakt1*.

**FIG. 2**     Analysis of *Dakt1* protein expression and activity in wild-type and *q* larvae.

**FIG. 3**     The phenotype of *Dakt1* germ line clone (GLC) mutants.

**FIG. 4**     Monitoring PCD markers in *Dakt1* GLC embryos.

**FIG. 5**     Phosphorylation of *trachealess* by PKB (I).

25    **FIG. 6**     Phosphorylation of *trachealess* by PKB (II).

**FIG. 7**     PKB specifically phosphorylates *trachealess* at Serine 665.

FIG. 8 Schematic representation of the *trachealess*/dARNT (*tango*) hetero-dimer transactivation of the *Drosophila* *breathless* gene and promotion of trachea development.

FIG. 9 The regulation of *trachealess*/dARNT transactivation via S665 of *trachealess*.

#### DETAILED DESCRIPTION OF THE FIGURES

5 FIG. 1 The screen focuses on the dominant enhancement of the zygotic *Dakt1* phenotype by second site lethal mutations. The source of these lethals can be selected from mutant stocks that can be generated either by exposing wild-type flies to ionizing radiation, chemical mutagenesis or P-element insertion into their genome. This screening approach benefits from the observation that the *Dakt1* mutant is a zygotic larval lethal that does not die during embryogenesis. The cross is performed with heterozygous *Dakt1*/+ females to the various recessive lethal mutant fly  
10 lines. Progeny will be analyzed for failure to hatch. Only interacting loci will generate dead embryos which will then be prepared for cuticle analysis. For example, fly lines with P-element mutations are tested for their ability to cause enhanced embryonic lethality in *Dakt1* mutant heterozygous embryos that are expected to develop normally (embryonic lethality of 0–1%). The P-elements and their induced mutants are stabilized on their respective chromosomes and maintained as stocks by being kept in *trans* to balancer chromosomes. The heterozygous  
15 embryos have only half the normal amount of maternal *Dakt1* (due to expression being limited to only one gene) and would also be carrying only one wild type copy of both *Dakt1* and the P-element mutant gene. If the mutant gene had a wild-type function in *Dakt1* signalling, it could be expected that loss of half of the maternally-contributed *Dakt1*, combined with the loss of half the normal zygotic expression of the mutant gene, might result in some lethality in the *trans*-heterozygotes. Enhancement is scored when a significant number of embryos fail to  
20 hatch and produce a phenotype.

FIG. 2 (A) Results of *Dakt1* protein expression and activity in wild-type and *q* larvae. (B) Immunoblot analysis of *Dakt1* protein from wild-type and *q* larvae using anti-*Dakt1* antibody. (C) Schematic diagram of *Dakt1* amino acid sequence showing the phenylalanine to isoleucine (F→I) change at amino acid 327 in the *q* allele of *Dakt1* and the sequence of the relevant region in the bovine and human PKB homologs. (D) Corresponding  
25 sequence change of F293 to I in bovine PKB creates a protein lacking kinase activity. Testing of the kinase activity and expression of PKB(F293I) mutant and PKB wt in HeLa S3 cells. "+" denotes addition of pervanadate.

FIG. 3 (A) First instar cuticle of wild-type larva. (B) Phenotype of *Dakt1* GLC embryo in the absence of zygotic *Dakt1* activity showing almost complete loss of cuticle. (C) Phenotype of *Dakt1* GLC embryo with zygotic *Dakt1* activity showing complete loss of head and dorsal structures. Only a portion of the ventral cuticle remains and does not show extensive defects in segmentation. (D) Phenotype of *Dakt1* GLC embryo rescued with Hs*Dakt1*  
30 induced maternally and zygotically. Note the reappearance of most cuticle structures demonstrating a functional rescue of the phenotype. (E) Acridine Orange (AO) satining of wild-type late stage 12 embryo. Note the signal present in the amnioserosa and in the regions of the head. (F) AO staining of stage 12 *Dakt1* GLC embryo showing ubiquitous AO signal.

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FIG. 4 (A) TUNEL signal in a stage 11 wild-type embryo. Only a few cells are positive for the TUNEL signal at this stage. (B) TUNEL signal in a stage 8 *Dakt1* GLC embryo. Even by stage 8, extensive DNA fragmentation as assayed by TUNEL has begun in the absence of *Dakt1*. (C) A stage 11 wild-type embryo showing the presence of Crq-expressing macrophages near sites of apoptosis (e.g. presumptive amnioserosa). (D,E) A stage 11 *Dakt1* GLC embryo showing the extensive and widespread presence of macrophages as assayed by Crq signal. (F) Close-up of (E). The expression of Rpr is not appreciably altered in (G) stage 10 *q* GLC embryos compared to (H) wild-type stage 10 embryos. (I) The cuticle phenotype of *Dakt1* GLC embryos homozygous for *H99* is similar to that of *Dakt1* mutants. (J) Cuticle phenotype of *Dakt1* GLC embryos expressing ectopic baculoviral p35 from *hs-p35* transgene. This indicates suppression of the *Dakt1* GLC phenotype by *p35* but not the *H99* deficiency.

FIG. 5 293 cells were transfected with nucleic acid sequences encoding either HA tagged PKB inactive kinase ( $\Delta$ , lane 1 and 4), or HA-tagged PKB wild-type (WT, lane 2,3,5, and 6). 48 hr after transfection, cells were treated with 50 ng/ml of IGF-1 (activation "+", lanes 1,3,4 and 6), or 100 nmol of wortmannin (activation "-", lanes 2 and 5) for 15 min. HA tagged PKB's were immunoprecipitated from cell lysates with anti-HA 12CA5 antibody. Both *trachealess* proteins, *Trh* N-term (1-175 aa) and *TrhI* (477-728 aa, the region that contains the two putative PKB phospho-serines, Ser 571 and Ser 665), were expressed in *E. coli* strain BL21 DE3 and purified as 6X Histidine fusion proteins over a Ni-agarose column. *Trachealess* proteins were subjected to PKB kinase assays in the presence of [ $\gamma$ - $^{32}$ P] ATP. The reaction mixtures were resolved on a 15% SDS-PAGE and  $\gamma$ - $^{32}$ P incorporation visualized on a Molecular Dynamics Phosphorimager.

FIG. 6 Phosphorylation of *trachealess* protein by PKB was performed essentially as described in FIG. 5. In this experiment, another fragment of the *trachealess* protein, *Trh II*, was tested as a substrate for PKB. The nucleic acid sequence for *TrhII* also encodes a fragment encompassing the two putative serine residues for PKB phosphorylation (amino acids 546-728). *TrhII* was also phosphorylated by PKB kinase activity (lane 6, top panel). The  $^{32}$ P incorporated *TrhII* can be stripped off by the addition of protein serine/threonine phosphatase (lane 7). The bottom panel is the Commassie Blue stain of the gel, showing that equal amount of *trachealess* protein (*trhI* vs. *trhII*) was added to each assay.

FIG. 7 Site specific mutations at Ser 571→Alanine (lanes 4-6), Ser 665→Alanine (lanes 7-9), or a double mutation (AA) at Ser 571→Alanine and Ser 665→Alanine (lanes 10-12) of *trachealess* protein were made by methods well known in the art. (Top panel) Phosphorylation of these substrates by active PKB was assayed as described above and compared with wild-type *trachealess* protein (lanes 1-3). Phosphorylation by PKB was abolished by Ser 665→Alanine and not surprisingly by the double mutation as well, establishing that PKB targets *trachealess* on Ser 665. The Bottom panel shows equal loading of the *trachealess* substrates.

FIG. 8 Genetic evidence from *Drosophila* analysis indicates that *trachealess*/dARNT heterodimer recognizes *Drosophila* *breathless* promoter/enhancer sequence and transactivates the expression of *breathless* gene. Hence, the



transactivation activity of *tracheless*, either by itself or by the partnership with *dARNT*, can be evaluated with the transactivation from *breathless* enhancer/promoter (B123).

FIG. 9 SL-2 cells were transfected with *tracheless* and/or *dARNT* (as indicated), together with a *breathless* promoter-luciferase reporter. 48 hr after transfection, cell lysates were subjected to luciferase assays. Both *tracheless* wild-type and Ser665→Asp (S665→D) mutant. (negatively charged Asp residue partially mimicks phosphoserine and gives constitutive activity of *tracheless*), as well as *dARNT* transactivate from *breathless* promoter. This transactivation is synergistically elevated by *tracheless*/*dARNT*, partnership. However, *tracheless*, either alone or in partnership with *dARNT*, contributes no transactivation activity to the reporter when the targeted Serine is mutated to Alanine (S665→A), an inactive mutation.

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#### DETAILED DESCRIPTION OF THE INVENTION

The invention herein describes a method for obtaining mutant *Drosophila melanogaster* flies with a mutation in a nucleic acid sequence known to disrupt PCD with an object of initiating a genetic analysis of the regulatory components of the PCD pathway. The invention herein further describes a genetic screen using a mutant fly to screen for other genes involved in the PCD pathway in *Drosophila* and to their encoded RNA and protein products. By extension, the mammalian counterparts to these genes will be components of PKB signalling in mammals and can be manipulated for the process of drug discovery for PKB related diseases or processes.

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#### Methods for obtaining and characterizing the nucleic acid sequence of *Drosophila melanogaster* mutants of *Dakt*

*Drosophila* mutants of *Dakt1*, can be identified using a lethal *Drosophila* fly line for failure to complement a large deletion at cytological position 89B but which can be rescued by a *Dakt* transgene. The lethal fly line is generated by exposing wild-type flies to either ionizing radiation, chemical mutagenesis or by P-element transformation. Since molecular analysis has placed *Dakt1* within *Df(3R)sbd<sup>15</sup>*, screening a collection of mutants mapping to *Df(3R)sbd<sup>15</sup>* can identify larval lethal mutations which can be subsequently rescued by a *Dakt1* transgene. Mutations in the nucleic acid sequence are thus identified by sequencing the coding region from genomic DNA isolated from the mutant larva and compared with the wild type sequence. Methods for sequencing nucleic acids are well known in the art and are described in Current Protocols in Molecular Biology 1995.

25

Alternatively, once known, one of ordinary skill in the art can engineer the mutation in the nucleic acid sequence using site-directed mutagenesis and by cloning into a pUAST transformation vector as described by Brand *et al. Methods Cell Biol.* 44: 635-654 After P-element transformation, the transgenic flies are to be crossed into a background of a transcript-null *Dakt1* mutant in combination with a ubiquitous promoter-*GAL4* transgene. The transcript-null *Dakt1* mutant is generated using X-ray mutagenesis and assayed using Southern blotting.

30

The *Drosophila* mutant of *Dakt1* described herein can also be obtained if mutations other than the one described herein of *Dakt1* are isolated and characterized as kinase dead point mutants. Such mutants should also provide a comparable allele to the one utilized herein.

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### Mutagenesis

Chemical mutagens include ethylmethanesulfonate (EMS), methylmethanesulfonate (MMS), methylnitrosoguanidine (NTG), 4-nitroquinoline-1-oxide (NQO), 2-aminopurine, 5-bromouracil, ICR 191 and other acridine derivatives, sodium bisulfite, ethidium bromide, nitrous acid, hydroxylamine, N-methyl-N'-nitroso-N-nitroguanidine, and alkylating agents. Physical mutagens include ultraviolet radiation and x-rays.

Mutagenesis is accomplished according to methods well-known in the art (see, e.g., Current Protocols in Molecular Biology 1995, Vol. 2, Section 13.3, wherein all the cited references are dated 1990 or earlier). Conditions for mutagenesis such as concentration (chemical mutagenesis) or intensity (e.g., ultraviolet mutagenesis) and duration are preferably optimized to produce a high rate of mutation while minimizing the amount of killing among the exposed flies. In general, mutagenesis is performed at a temperature that is below the optimal growing temperature for the organism, because the sub-optimal temperature has been found to decrease cell killing.

P-elements are transposable genetic elements that generate mutations by insertion into different parts of the *Drosophila* genome. The P-elements and their induced mutants are stabilized on their respective chromosomes and maintained as stocks by being kept in *trans* to balancer chromosomes. Methods for generating mutant flies with P-element insertions are well known in the art and are described in (*Drosophila*, a Laboratory Handbook, Michael Ashburner, Cold Spring Harbor Laboratory Press, 1989).

### The genetic screen

The screen focuses on the dominant enhancement of the zygotic *Dakt1* phenotype by second site lethal mutations or "lethals". The source of these lethals can be selected from mutant stocks that can be generated either by exposing wild-type flies to ionizing radiation, chemical mutagenesis or P-element insertion into their genome. Generally, embryonic screens are not undertaken due to the success of eye screens (Doyle *et al. Genes & Dev.* 7, 633-646; Verheyen *et al. Genetics* 144, 1127-1141; Karim *et al. Genetics* 143, 315-329; Dickson *et al. Genetics* 142, 163-171; Ma *et al. Genetics* 142, 1199-1213). However, to date, it has not been possible to generate a *Dakt1* overexpression or dominant-negative phenotype in the eye which can be easily scored in a screen. Thus, the embryonic scheme is the only one possible at this time. This screening approach benefits from the observation that the *Dakt1* mutant is a zygotic larval lethal that does not die during embryogenesis. This is because in *Dakt1* homozygous mutant embryos the maternal contribution of *Dakt1* from the heterozygous mother is only half the amount that would be contributed by a wild-type mother, but the homozygous embryos still have enough maternally-derived *Dakt1* to rescue them to 1st or 2nd instar larvae before dying. Prior to this point during development, the requirement for zygotically-expressed *Dakt1* has been masked by the presence of the maternally-derived enzyme. It is, presumably, at this stage of development when zygotic expression becomes necessary, possibly due to a gradual loss of the maternally derived enzyme during development. Heterozygous embryos are viable and develop into normal larvae, pupae and adults.

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The cross is performed with heterozygous *Dakt1*<sup>+</sup> females to the various recessive lethal mutant lines generated by exposing wild-type flies to ionizing radiation, chemical mutagenesis or by P-element insertions. The progeny will be analyzed for failure to hatch (see Figure 1 for screen schematic). Only interacting loci will generate dead embryos which will then be prepared for cuticle analysis. The inventors have observed that the extent of development of cuticle is a good first indicator for ectopic PCF. Staverley *et al. Current Biology*, 1998 8:599-602. For example, fly lines with P-element mutations are tested for their ability to cause enhanced embryonic lethality in *Dakt1* mutant heterozygous embryos that are expected to develop normally (embryonic lethality of 0-1%). The heterozygous embryos have only half the normal amount of maternal *Dakt1* (due to expression being limited to only one gene) and would also be carrying only one wild type copy of both *Dakt1* and the P-element mutant gene. If the mutant gene had a wild-type function in *Dakt1* signalling, it could be expected that loss of half of the maternally-contributed *Dakt1*, combined with the loss of half the normal zygotic expression of the mutant gene, might result in some lethality in the *trans*-heterozygotes. Enhancement is scored when a significant number of embryos fail to hatch and produce a phenotype.

Mutants which are positive in the screen are analyzed as follows. Mutants are mapped (meiotically or with deficiencies) and organized according to polytene chromosomal location. P-element induced alleles will be the first to be analyzed molecularly. However, with the rapid progress of the Berkeley *Drosophila* Genome Project (BDGP), many of the chemically induced mutants may be within molecular markers and thereby analyzable at the molecular level by searching the Berkeley Blast Server using the BLASTN algorithm Altschul *et al. J. Mol. Biol.* (1990) 215:403-10. For each new mutant complementation group, a series of genetic is carried out: (1). Interactions outlined in the screens are repeated to confirm the results. (2). Additional alleles can be identified or generated. (3). The embryonic phenotype of each mutant is assessed for possible involvement in development and ectopic apoptosis by terminal deoxy-transferase mediated hapten-labelled-dUTP nick end labelling (TUNEL) and acridine orange staining. (4). GLC analysis is used to assess the maternal effect phenotype of the mutants. (5). Where possible, genetic epistatic tests can be performed with *Dakt1*. Enhancers of *Dakt1* can be tested with activated *Dakt1* (e.g. CAAX and DD mutants) and suppressors can be tested with the *Dakt1* mutants.

#### Biological Functional Equivalents

Modification and changes may be made in the structure of the encoded polypeptides used in the vectors and DNA segments of the present invention and still obtain a functional molecule that encodes a protein or peptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following codon table:

TABLE 1

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Amino Acid	3 Letter Code	2 Letter Code	Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU		
Cysteine	Cys	C	UGC	UGU				
Aspartic Acid	Asp	D	GAC	GAU				
Glutamic Acid	Glu	E	GAA	GAG				
Phenylalanine	Phe	F	UUC	UUU				
Glycine	Gly	G	GGA	GGC	GGG	GGU		
Histidine	His	H	CAC	CAU				
Isoleucine	Ile	I	AUA	AUC	AUU			
Lysine	Lys	K	AAA	AAG				
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
Methionine	Met	M	AUG					
Asparagine	Asn	N	AAC	AAU				
Proline	Pro	P	CCA	CCC	CCG	CCU		
Glutamine	Gln	Q	CAA	CAG				
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	
Threonine	Thr	T	ACA	ACC	ACG	ACU		
Valine	Val	V	GUA	GUC	GUG	GUU		
Tryptophan	Trp	W	UGG					

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Tyrosine            Tyr            Y                    UAC    UAU

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For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that determines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence. and nevertheless obtain a protein with like properties. It is thus contemplated by the inventors that various changes may be made in the peptide sequences of the apoptotic proteins, or corresponding DNA sequences which encode said proteins with retention of biological utility or activity.

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Pat. No. 4,554,101, incorporated herein by reference, states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U.S. Pat. No. 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0,  $\pm 1$ ); glutamate (+3.0,  $\pm 1$ ); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5,  $\pm 1$ ); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

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It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those which are within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like (agonistic) properties. Equally, the same considerations may be employed to create a protein or polypeptide with countervailing (e.g., antagonistic) properties. It is thus contemplated by the inventors that various changes may be made in the sequence of the *Dakt1*, *trachealess* or *DPP24* protein or peptide (or underlying DNA) without appreciable loss of biological utility or activity.

#### Nucleic Acid Compositions

The scope of the invention with respect to nucleic acid compositions includes, but is not necessarily limited to, nucleic acids having a sequence set forth in any one of the sequences demonstrated by the present methods to be active in enhancing PCD; nucleic acids that hybridize the provided sequences under stringent conditions; genes corresponding to the provided nucleic acids; variants of the provided nucleic acids and their corresponding genes, particularly those variants that retain a biological activity of the encoded gene product. Other nucleic acid compositions contemplated by and within the scope of the present invention will be readily apparent to one of ordinary skill in the art when provided with the disclosure here.

The nucleic acids of the invention also include nucleic acids having sequence similarity or sequence identity. Nucleic acids having sequence similarity are detected by hybridization under low stringency conditions, for example, at 50°C and 1.0XSSC (0-9 M NaCl/0.09 M sodium citrate) and remain bound when subjected to washing at 55°C in 1.0XSSC. Sequence identity can be determined by hybridization under stringent conditions, for example, at 50°C or higher and 0.1XSSC (9 mM NaCl/0.9 mM sodium citrate). Hybridization methods and conditions are well known in the art. Nucleic acids that are substantially identical to the provided nucleic acid sequences, e.g. allelic variants, genetically altered versions of the gene, etc., bind to the provided nucleic acid sequences under stringent hybridization conditions. By using probes, particularly labelled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes can be any species.

Preferably, hybridization is performed using at least 15 contiguous nucleotides of at least one of the PCD enhancing sequence. The probe will preferentially hybridize with a nucleic acid or mRNA comprising the complementary sequence, allowing the identification and retrieval of the nucleic acids of the biological material that uniquely hybridize to the selected probe. Probes of more than 15 nucleotides can be used, e.g. probes of from about 18 nucleotides to not more than about 100 nucleotides, but 15 nucleotides generally represents sufficient sequence for unique identification.

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The nucleic acids of the invention also include naturally occurring variants of the nucleotide sequences, e.g. degenerate variants, allelic variants, etc. Variants of the nucleic acids of the invention are identified by hybridization of putative variants with nucleotide sequences disclosed herein, preferably by hybridization under stringent conditions. For example, by using appropriate wash conditions, variants of the nucleic acids of the invention can be identified where the allelic variant exhibits at most about 25–30% base pair mismatches relative to the selected nucleic acid probe. In general, allelic variants contain 5–25% base pair mismatches, and can contain as little as even 2–5%, or 1–2% base pair mismatches, as well as a single base-pair mismatch.

The invention also encompasses homologs corresponding to the provided nucleic acids, where the source of homologous genes can be any related species within the same genus or group. Within a group, homologs have substantial sequence similarity, e.g. at least 75% sequence identity, usually at least 90%, more usually at least 95% between nucleotide sequences. Sequence similarity is calculated based on a reference sequence, which may be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, etc. A reference sequence will usually be at least about 18 contiguous nucleotides long, more usually at least about 30 nucleotides long, and may extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul *et al.*, *J. Mol. Biol.* (1990) 215:403–10.

In general, variants of the invention have a sequence identity greater than at least about 65%, preferably at least about 75%, more preferably at least about 85%, and can be greater than at least about 90% or more as determined by the Smith-Waterman homology search algorithm as implemented in MPSRCH program (Oxford Molecular). For the purposes of this invention, a preferred method of calculating percent identity is the Smith-Waterman algorithm, using the following. Global DNA sequence identity must be greater than 65% as determined by the Smith-Waterman homology search algorithm as implemented in MPSRCH program (Oxford Molecular) using an affine gap search with the following search parameters: gap open penalty, 12; and gap extension penalty, 1.

The subject nucleic acids can be cDNAs or genomic DNAs, as well as fragments thereof, particularly fragments that encode a biologically active gene product and/or are useful in the methods disclosed herein. The term "cDNA" as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons and 3' and 5' non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns, when present, being removed by nuclear RNA splicing, to create a continuous open reading frame encoding a polypeptide of the invention.

A genomic sequence of interest comprises the nucleic acid present between the initiation codon and the stop codon, as defined in the listed sequences, including all of the introns that are normally present in a native chromosome. It can further include the 3 and 5 untranslated regions found in the mature mRNA. It can further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, etc., including about 1 kb, but possibly more, of flanking genomic DNA at either the 5 and 3 end of the transcribed region. The genomic DNA can be isolated as a fragment of 100 kbp or smaller, and substantially free of flanking chromosomal

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sequence. The genomic DNA flanking the coding region, either, 3' and 5', or internal regulatory sequences as sometimes found in introns, contains sequences required for expression.

5 The nucleic acid compositions of the subject invention can encode all or a part of the subject differentially expressed polypeptides. Double or single stranded fragments can be obtained from the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, etc. Isolated nucleic acids and nucleic acid fragments of the invention comprise at least about 15 up to about 100 contiguous nucleotides, or up to the complete sequence provided. For the most part, fragments will be of at least 16 nt, usually at least 18 nt or 26 nt, and up to at least about 50 contiguous nt in length or more.

10 Probes specific to the nucleic acids of the invention can be generated using the nucleic acid sequences disclosed, and the fragments as described above. The probes can be synthesized chemically or can be generated from longer nucleic acids using restriction enzymes. The probes can be labelled, for example, with a radioactive, biotinylated, or fluorescent tag. Preferably, probes are designed based upon an identifying sequence of a nucleic acid. More preferably, probes are designed based on a contiguous sequence of one of the subject nucleic acids that remain unmasked following application of a masking program for masking low complexity (e.g., XBLAST) to the 15 sequence, i.e. one would select an unmasked region, as indicated by the nucleic acids outside the poly-n stretches of the masked sequence produced by the masking program.

The nucleic acids of the subject invention are isolated and obtained in substantial purity, generally as other than an intact chromosome. Usually, the nucleic acids, either as DNA or RNA, will be obtained substantially free of other naturally-occurring nucleic acid sequences, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", e.g., flanked by one or more nucleotides with which it is not normally associated on 20 a naturally occurring chromosome.

The nucleic acids of the invention can be provided as a linear molecule or within a circular molecule. They can be provided within autonomously replicating molecules (vectors) or within molecules without replication sequences. They can be regulated by their own or by other regulatory sequences, as is known in the art. The nucleic 25 acids of the invention can be introduced into suitable host cells using a variety of techniques which are available in the art, such as transferrin polycation-mediated DNA transfer, transfection with naked or encapsulated nucleic acids, liposome-mediated DNA transfer, intracellular transportation of DNA-coated latex beads, protoplast fusion, viral infection, electroporation, gene gun, calcium phosphate-mediated transfection, and the like.

30 The subject nucleic acid compositions can be used to, for example, produce polypeptides, as probes for the detection of mRNA of the invention in biological samples (e.g., extracts of cells) to generate additional copies of the nucleic acids, to generate ribozymes or antisense oligonucleotides, and as single stranded DNA probes or as triple-strand forming oligonucleotides. The probes described herein can be used to, for example, determine the presence or absence of the nucleic acid sequences or variants thereof in a sample. The probes described herein can also be used for detection of genomic nucleic acid sequences and complementary DNA sequences from genomic



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nucleic acid and complementary DNA libraries. Methods of probing and generating such libraries are well known in the art and are described in Current Protocols in Molecular Biology 1995.

#### Programmed Cell Death Assay

5 The assay for PCD can be accomplished by methods well known in the art. The vital dyes acridine orange (AO), a fluorescent dye, and Nile Blue (NB) can be used to visualize programmed cell deaths in live *Drosophila* embryos. The dyes have a selective affinity for dying cells and are able to penetrate the tissues of the embryo. The dyes have been found to stain apoptotic cell corpses inside engulfing phagocytes, without staining the phagocytes themselves. The assay requires preliminary removal of the embryonic chorion with bleach, followed by permeabilization of the waxy coat with heptane or octane. The stained cells are then visualized by conventional  
10 microscopy and Nomarski optics, in the case of NB, or fluorescent microscopy, in the case of AO. Tissue sections can also be prepared from the stained embryos to obtain higher resolution of the cell structures.

AO was found to selectively stain apoptotic cells, whereas NB stains both apoptotic and necrotic cells. Since the vast majority of programmed cell deaths are apoptotic, both vital dyes were found to be useful for visualizing programmed cell deaths.

15 Using this assay, it was possible to determine the extent and pattern of ectopic PCD occurring throughout *Drosophila* embryogenesis, including those affecting the development of certain organs and body systems so as to quantitatively assay which embryos have undergone ectopic PCD.

#### Recombinant Host Cells, Vectors and their Promoters/Enhancers

20 As used herein, the term "engineered" or "recombinant" cell is intended to refer to a cell into which a recombinant nucleic acid sequence, such as a nucleic acid sequence encoding a protein identified by the genetic screen described above, has been introduced. Therefore, engineered cells are distinguishable from naturally occurring cells which do not contain a recombinantly introduced gene. Recombinantly introduced nucleic acid sequences will either be in the form of a cDNA nucleic acid sequence (i.e., they will not contain introns), a copy of a genomic nucleic acid sequence, or will include nucleic acid sequences positioned adjacent to a promoter not  
25 naturally associated with the particular introduced nucleic acid sequence.

The term promoter is used herein to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of  
30 discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins. At least one module in each promoter functions to position

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the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV 40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

5 Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to  
10 activate transcription.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each  
15 of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational  
20 distinction, enhancers and promoters are very similar entities. They have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

25 Generally speaking, it may be more convenient to employ as the recombinant nucleic acid sequence a cDNA version of the nucleic acid sequence. It is believed that the use of a cDNA version will provide advantages in that the size of the nucleic acid sequence will generally be much smaller and more readily employed to transfect the targeted cell than will a genomic nucleic acid sequence, which will typically be up to an order of magnitude larger than the cDNA nucleic acid sequence. However, the inventors do not exclude the possibility of employing a  
30 genomic version of a particular nucleic acid sequence where desired.

The engineering of DNA segment(s) for expression in a prokaryotic or eukaryotic system may be performed by techniques generally known to those of skill in recombinant expression. It is believed that virtually any expression system may be employed in the expression of the PCD nucleic acid sequence identified. In general, plasmid vectors containing replicon and control sequences which are derived from species compatible with the host

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cell are used in connection with these hosts. The vector ordinarily carries a replication site, as well as marker sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (Bolivar *et al.*, 1977). pBR322 contains nucleic acid sequences for ampicillin and tetracycline resistance and thus provides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters which can be used by the microbial organism for expression of its own proteins.

In addition, phage vectors containing replicon and control sequences that are compatible with the host microorganism can be used as transforming vectors in connection with these hosts. For example, the phage lambda GEM.TM.-11 may be utilized in making a recombinant phage vector which can be used to transform host cells, such as *E. coli* LE392.

Those promoters most commonly used in recombinant DNA construction include the B-lactamase (penicillinase) and lactose promoter systems (Chang *et al.*, 1978; Itakura *et al.*, 1977; Goeddel *et al.*, 1979) and a tryptophan (trp) promoter system (Goeddel *et al.*, 1980; EPO Appl. Publ. No. 0036776). While these are the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (EPO Appl. Publ. No. 0036776).

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used. *Saccharomyces cerevisiae*, or common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb *et al.*, 1979; Kingsman *et al.*, 1979; Tschemper *et al.*, 1980). This plasmid already contains the trp1 nucleic acid sequence which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzeman *et al.*, 1980) or other glycolytic enzymes (Hess *et al.*, 1968; Holland *et al.*, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these nucleic acid sequences are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Any

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plasmid vector containing a yeast-compatible promoter, an origin of replication, and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate cells in culture (tissue culture) has become a routine procedure in recent years (Tissue Culture, 1973). Examples of such useful host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7, 293 and MDCK cell lines. Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front of the nucleic acid sequence to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

#### DNA Delivery

Following the identification of novel regulatory components by the method of the invention described herein, it is envisioned that the present invention may generally next be used to direct the introduction an exogenous nucleic acid sequence, such as a cDNA or gene, into a recipient cell to create a transformed cell. The frequency of occurrence of cells receiving exogenous nucleic acid sequences is believed to be low. Moreover, it is most likely that not all recipient cells receiving nucleic acid sequences will result in a transformed cell wherein the nucleic acid is stably integrated into the animal genome and/or expressed. Some may show only initial and transient gene expression. However, certain eukaryotic cells may be stably transformed, and these cells developed into transgenic animals, or arthropods, preferably insects.

There are many methods for introducing transforming nucleic acid sequences into cells, but not all are suitable for delivering nucleic acid sequences to eukaryotic cells. Suitable methods are believed to include virtually any method by which nucleic acid sequences can be introduced into a cell, such as by direct delivery of nucleic acid sequences, by desiccation/inhibition-mediated nucleic acid sequences uptake, by electroporation, by agitation with silicon carbide fibers, by acceleration of nucleic acid sequences coated particles, by chemical transfection, by lipofection or liposome-mediated transfection, by calcium chloride-mediated nucleic acid sequences uptake, etc. In certain embodiments, acceleration methods are preferred and include, for example, microprojectile bombardment and the like.

#### Pharmaceutical Assays and Methods for Identifying Drug Candidates

This invention also provides pharmaceutical assays and methods for identifying drug candidates, i.e. chemical compounds which may be useful as drugs for the treatment of PCD related diseases. Such assays and methods involve testing compounds for their effects on the activity of the regulatory component identified by the method of the invention described herein, the mammalian counterparts of which will be components of PKB

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signalling in mammals. Examples of effects for which testing may be conducted include inhibition, activation or enhancement of enzymatic activity, e.g. kinase activity of *Dakt1* or phosphatase activity of *DPP2A*.

Thus, drugs that elevate expression of wild-type *Dakt1* or *DPP2A* or their mammalian counterparts, PKB or PP2A; inhibit expression of a mutated or altered form of these proteins or inhibit a protein that antagonizes the activity of these proteins may be useful in treating diseases in which it is preferred that PCD be inhibited. Further, RNA or DNA probes may be used to detect chemical agents that alter expression or activity of the proteins. Moreover, the *Dakt1* or *DPP2A* nucleic acids may also be used to engineer cells that would be useful for the detection of chemicals that alter expression or activity of the proteins. Most preferably, the *Dakt1* or *DPP2A* protein may be used in direct biochemical assays for agents that activate or alter its activity. If one of the components identified by the genetic screen described herein is a transcription factor, reporter assays can be designed for identifying agents which can modulate *Dakt1* expression. For example, host cells known to contain *Dakt1* or PKB can be transfected with a plasmid reporter gene, wherein the reporter gene (*E. coli lac Z*, or luciferase) is fused to the promoter/enhancer module of the transcription factor known to be in the *Dakt1*/PKB pathway. The cells are next exposed to an agent for a period of time, and the level of reporter gene product expressed is compared the level of reporter gene product expressed in cells not exposed to the drug (reference cell). If the reporter gene product expressed in the cells exposed to the agent is higher when compared to the level of the reporter gene product in the reference cells, then the agent is able to enhance expression of *Dakt1*/PKB or maintain *Dakt1*/PKB in a constitutively active state. Such agents will be useful in treating diseases where it is desirable to inhibit PCD. Conversely, identification of agents capable of inhibiting expression of the reporter gene product can also be assayed by this method for the identification of agents for inducing PCD.

An alternative method for determining the effect of an agent on the activity of *Dakt1*/PKB is to assay for the level of product of the natural gene fused to the promoter/enhancer module to which the transcription factor binds and initiates transcription. For example, it is known that the protein product of *trachealess* initiates transcription of the *breathless* gene by contacting the B123 promoter/enhancer module. Instead of engineering a reporter gene construct, the effect of an agent on the activity of *trachealess* can be determined by assaying the level of *breathless* DNA by RT-PCR produced in the cell exposed to the agent for a period of time and comparing it to the level of *breathless* DNA produced by RT-PCR in a cell not exposed to the agent.

In vitro studies may use purified target macromolecules to screen large compound libraries for inhibitory drugs; or the purified target molecule may be used for a rational drug design program, which requires first determining the structure of the macromolecular target or the structure of the macromolecular target in association with its customary substrate or ligand. This information is then used to design inhibitory compounds which must be synthesized and tested further. Test results are used to refine the molecular models and drug design process in an iterative fashion until a lead compound emerges.

Kinases, particularly protein kinase B and homologs thereof are involved in multiple biologically important processes. Pharmacological agents designed to affect only specific kinase activities are of particular interest.

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Presently available compounds tend to be non-specific and elicit both positive and negative responses, thereby reducing clinical efficacy. The subject mutations may be used in in vitro and in vivo models to test the specificity of novel compounds, and of analogs and derivatives of compounds known to act on kinases. Numerous pharmacological agents have profound affects on kinase activity.

5           Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including monitoring cellular excitation and conductance, labelled in vitro protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modelling intermolecular interactions.

10           The term "substance" or "agent" as used herein describes any molecule, e.g. protein or pharmaceutical. With the capability of inhibiting, altering or mimicking the kinase activities associated with the screened gene products. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the level of detection.

15           Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more then 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbanyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups.  
20           Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

          Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of  
25           organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as  
30           acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

          Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescence, chemiluminescence, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific

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binding members, the complementary member would normally be labelled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc that are used to facilitate optimal protein-protein binding and/or reduce non-specific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc. may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4 and 40°C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

10

#### Example 1

Molecular analyses localized *Dakt1* to within ~30 kb of the *stubbloid* (*sbd*) gene (Franke *et al. Oncogene* 1994, 9: 141-148; Andjelkovic *et al. J. Biol. Chem.* 1995, 270: 4066-4075) placing *Dakt1* within *Df(3R)sbd<sup>Δ5</sup>* (Appel *et al. Proc. Natl. Acad. Sci. USA* 1993, 90: 4937-4941). Thus, a hunt for a *Dakt1* mutant focused on lethals that failed to complement a large deletion at cytological position 89B on the right arm of the third chromosome of *Drosophila melanogaster*. As *Dakt1* is ubiquitously expressed (Franke *et al. Oncogene* 1994, 9: 141-148; Andjelkovic *et al. J. Biol. Chem.* 1995, 270: 4066-4075), a *GAL4* transgene driven by the *armadillo* promoter (*arm-GAL4*) was used to allow expression of *Dakt1* in a fairly ubiquitous pattern using an upstream activating sequence *Dakt1* (*UAS-Dakt1*) transgene. Screening a collection of mutants mapping to *Df(3R)sbd<sup>Δ5</sup>*, one larval lethal mutation, *l(3)89Bq<sup>1</sup>* was identified that could be rescued by the combination of the *arm-GAL4* and *UAS-Dakt1* transgenes. *arm-Dakt1* (% rescue=34.1). As PKB/AKT and *Dakt1* have a similarity of 76.5% at the amino-acid level [(Franke *et al. Oncogene* 1994, 9: 141-148; Andjelkovic *et al. J. Biol. Chem.* 1995, 270: 4066-4075; Coffey *et al. Eur. J. Biochem.* 1991, 201: 475-481), bovine PKB/AKT was tested for its ability to rescue *l(3)89Bq<sup>1</sup>* mutants using a *UAS-PKB/AKT* transgene. The *arm-PKB/AKT* combination was effective at rescuing *l(3)89Bq<sup>1</sup>* albeit with a lower efficiency (% rescue=17.8), demonstrating that *l(3)89Bq<sup>1</sup>* or simply 'q' encodes *Dakt1*, the functional homolog of PKB/AKT. Rescue was scored as the percentage of flies with a homozygous *q* chromosome. For Both *Dakt1*, bovine PKB, and PKB DD mutant cDNAs were cloned into pUAST P-element transformation vector and injected into *w<sup>1118</sup>* embryos to generate transformants. The heat-shock inducible *HsDakt1* line was generated using the pHS Casper P-element vector. For the rescue experiments, the following crosses were made: (A) *q/TM6B=q<sup>1</sup>/TM6B Tb e Hu* (NX-) (B) (*arm-Dakt1*) *q/TM6B=armGAL4; q<sup>1</sup>/TM6B Tb e Hu* X *UASDakt1/CyO; q<sup>1</sup>/TM6B Tb e Hu*. The results of cross B were corrected for the *CyO* chromosome where *CyO* progeny (which would not carry *UASDakt1*) were removed from the data. (C) (*arm-PKB*) *q/TM6B=GAL4; q/TM6B Tb e Hu* X *UASPKB; q<sup>1</sup>/TM6B Tb e Hu*. (D) (*HsDakt1*) *q/TM6B=HsDakt1; q<sup>1</sup>/TM6B Tb e Hu* (NX-). For heat-induced rescue, flies were heat-shocked 30 min daily after egg laying in glass vials.

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GLC analysis for 3R using *FRT<sup>82B</sup>* was performed as described in (Chou *Et al. Genetics* 1996, 144: 1673-1679). For determining the extent of zygotic/paternal rescue, *l(3)89Bq<sup>1</sup>* GLC females were crossed to wild-type, *l(3)89Bq<sup>1</sup>*, *TM3*, *Sb*, *l(3)89B q<sup>2</sup>* and *Df(3R)sbd<sup>45</sup>* males and the embryonic phenotypes compared. As *l(3)89Bq<sup>1</sup>* and *Df(3R)sbd<sup>45</sup>* chromosomes behaved identically, it is believed that *l(3)89Bq<sup>1</sup>* is a genetically null allele of *Dakt1*. Also a second *Dakt1* allele (termed *q<sup>2</sup>*) was also used for these experiments. However, as *q<sup>2</sup>* was induced on *TM3* chromosome, it was not possible to perform GLC experiments using this allele.

*Df(3L)H99 FRT<sup>82B</sup> l(3) 89B q<sup>1</sup>* chromosome was constructed using standard recombination techniques well known in the art and then crossed to *FRT<sup>82B</sup> ovoD* males for the induction of GLCs. GLC females were then crossed to the *Df(3L)H99 FRT<sup>82B</sup> l(3) 89B q<sup>1</sup>* chromosome and the progeny analyzed.

A 1 kb *Bam*HI fragment of pBSPS containing the entire coding region of *Autographica californica* P35 (Clem *et al. Mol. Cell. Biol.* 1994, 14: 5215-5222) was cloned into the *Bgl*II site of pHS Casper and transformed. For the epistasis experiment, *q<sup>1</sup>* GLC females were crossed to HsP35; *q<sup>1</sup>/TM3* males and the progeny collected onto nylon sieves. Embryos were then staged (stages 10-12), heat-shocked at 37°C in a water bath for 8 min and allowed to recover at 25°C. Embryos were then allowed to develop cuticle and analyzed.

For ectopic expression of activated PKB (PKB DD), *armGAL4* females were crossed to *UAS PKB DD* males to generate *arm-PKB DD* embryos. Embryos were then collected and either treated for Acridine Orange (AO) staining or for antibody staining.

### Example 2

Antisera against *Dakt1* were raised against a histidine tagged *Dakt1* fusion protein (His-*Dakt1*). His-*Dakt1* was constructed by cloning a 1590 bp fragment of the *Dakt1* complete coding region (530 amino acids) into the *Bam*HI site of the pET 15b vector (Novagen). Fusion proteins were produced in *E. coli* strain (BL21) and purified from bacterial lysates through binding to a nickel-chelating resin according to the manufacturer's instructions (Novagen). Rabbits were immunized subcutaneously with purified recombinant proteins in complete Freund's adjuvant followed by booster injections at 4 week intervals. For kinase assays, *Dakt1* and its mutant, *Dakt1<sup>q</sup>*, was immunoprecipitated using the antibody to *Dakt1* generated as described above from lysates of embryos prepared by lysing the embryos in Gentle Soft buffer (20mM PIPES pH 7.4, 10mM NaCl, 0.5% NP-40, 5mM EDTA, 0.05% 2-mercaptoethanol, 5mg/ml leupeptin, 1mM benzamidine, 0.5 mM NaF and 100 mM Na vanadate). Larvae homozygous for *q* (expressing *Dakt1<sup>q</sup>*) were identified by following the TM6B balancer chromosome. Lysates were normalized for total protein before immunoprecipitation. 5 ml of rabbit polyclonal antiserum were added to the cell lysates for 2 h at 4°C. Immunocomplexes prebound to protein A-Sepharose (Sigma) were washed five times with lysis buffer prior to performing the kinase assays. *In vitro* *Dakt1*, *Dakt1<sup>q</sup>* (FIG. 1 (A)). HA tagged bovine wild-type PKB and kinase inactive HA-PKB F293I (FIG. 1 (D)) kinase assays were measured by the capacity of the kinases to phosphorylate a GSK3 peptide substrate corresponding to the sequence in GSK3β surrounding the Ser9 site that is



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phosphorylated by PKB. The *Dakt1*, *Dakt1<sup>q</sup>* and HA tagged bovine PKB immunocomplexes were incubated at 30°C with 30mM GSK3 peptide in the presence of 50 mM [ $\gamma$ -<sup>32</sup>P] ATP in 50 mM PIPES, pH 7.4, 10 mM MgCl<sub>2</sub> and 1 mM EGTA. The phosphorylated peptides were separated from unincorporated [ $\gamma$ -<sup>32</sup>P] ATP by Tricine-SDS-PAGE and quantified by analysis on a Molecular Dynamics Phosphorimager.

- 5 For immunoblot analysis, lysates were prepared as described above and normalized for total protein before separation by SDS-PAGE for immunoblotting. *Dakt1* and its mutant, *Dakt1<sup>q</sup>* protein was visualized using the ECL system (Amersham) (FIG. 1 (B)).

### Example 3

- 10 A genetic screen as described above was performed against a collection of P-element induced mutant fly lines as well as other lethals with the intention of identifying genes that interact with *Dakt1*. The inventors discovered that a mutant fly line, P1747, resulted in an average of 7.4% lethality while in *trans* to *Dakt1*. This mutant has a P-element insertion in the 5' untranslated region of the *trachealess* (*trh*) gene (SEQ. ID No. 1) at cytological region 61C of chromosome three. Surprisingly no ectopic PCD was associated with the dead embryos from the *trh/Dakt1* cross when assayed for PCD by the standard methods described above. This suggested that
- 15 *Dakt1*'s involvement with the *trh* protein (SEQ. ID No. 2) may be separate from its role in PCD and follows that *Dakt1* may be involved in *trh*-mediated pathways as well. Inspection of the amino acid sequence of *trh* protein showed that it contains two consensus sequences for PKB phosphorylation. The first is amino acid sequence RGRGRS<sup>571</sup>AA in which Ser 571 may be phosphorylated. The second is amino acid sequence RSRLPS<sup>665</sup>IV in which Ser 665 may be phosphorylated.

- 20 To determine if *trachealess* protein is a substrate for PKB kinase activity, a kinase assay was performed as described in Example 2 and in the detailed description of FIG. 5 with PKB as the kinase and *trachealess* protein or fragments thereof as the test substrates. FIG. 5 shows that only *Trhl*, containing the two putative serine residues is phosphorylated by active PKB and not kinase inactive PKB or wild-type PKB inactivated by the PI3'K inhibitor wortmannin. These data validate *trachealess* protein as a substrate for PKB. The data presented in FIG. 6 and FIG.
- 25 7 confirm PKB phosphorylation on Ser 665 of the *trachealess* protein fragment substrates.

- In summary, these data show an unexpected result obtained from the genetic screen described above. The identification of dead embryos not undergoing ectopic PCD was a complete surprise considering that the genetic screen described above is designed to identify enhancers of the zygotic *Dakt1* phenotype, i.e. enhanced PCD by second site lethal mutations. Although the embryos scored, were dead, ectopic PCD was not observed. Instead it
- 30 was determined that death was a result of abnormal embryonic development, thus indicating that *Dakt1*'s involvement with *trachealess* is separate from its role in apoptosis. Such a role for *Dakt1* or its mammalian homolog, PKB, has not been identified in the prior art.

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*Trachealess* is a master regulatory gene that induces differentiation of the embryonic tracheal system from ectodermal precursor cells in *Drosophila*. At stage 10 (4 hours after egg lay [AEL]) of embryonic development, clusters of cells on both sides of each of the ten posterior parasegments (T2-A8) assume a tracheal-specific fate. During stages 10 and 11 (4-5h AEL), each cluster of precursor cells undergoes two postblastoderm mitoses: the first mitosis event establishes a region in the ectoderm called the tracheal placode, and the second mitosis event is coincident with invagination of the tracheal placode and formation of the tracheal pit. Subsequent formation of the tracheal system occurs by cell migration and shape changes without further cell divisions. Tracheation occurs in two general stages, primary and terminal tracheation. Primary tracheation (stages 11-16 [5h AEL-hatching]) is the establishment of the stereotypical branching pattern of the main multicellular and unicellular tracheae that make up each tracheal metamere. Terminal tracheation begins during stage 15 (11-13h AEL) and continues into the larval period. This overlapping stage is characterized by the development of fine unicellular and subcellular tracheae that branch from, or are continuations of, the main components of the tracheal system. The terminal branches are extensive arrays of subcellular tubes that reach individual target cells or tissues in the embryo. The pattern of primary branching is stereotypical but the pattern of terminal branching is highly variable and most likely dependent on the oxygen needs of target tissues. The entire tracheal network in the embryo is continuous and supplied by a single opening, the posterior spiracle, which forms during stage 11 (5h AEL) by fusion of the tracheal pit and posterior spiracle in parasegment A8.

Coincident with the establishment of tracheal cell fate at stage 10 is the expression of *Trh* and *Vvl* (Ventral Veinless/Drifter) in the tracheal precursor cells. Immunostaining has shown that *Trh* protein is localized to the nuclei of the tracheal precursors and that, once initiated, it is expressed for the remainder of embryogenesis and throughout most of larval development. The *Trh* protein is required for the initial invagination of the tracheal placodes, tubulogenesis and the expression of tracheal-specific genes. Despite its early co-expression with *Trh*, the *Vvl* POU domain-containing transcription factor is needed later in development for migration of the primary branches and the expression of some tracheal-specific markers, but it is not required for any initiation events. The segment polarity genes *wg* (wingless) and *dpp* (decapentaplegic) appear to have an effect on the localization of the *Trh* signal in the parasegment, and the homeotic genes may also play a role in determining the initial spatial expression of *Trh* since this gene is only expressed in ten of the fourteen parasegments.

*Trh* is a member of the bHLH-PAS family of transcription factors. The 949 amino acid protein contains a bHLH (basic helix-loop-helix), PAS (homology to *Per*, *Arnt* and *Sim*) and transcription activation domains, as well as a nuclear localization signal (NLS). The bHLH domain determines the protein's ability to contact DNA and form dimers, while no function has been attributed to the PAS domain.

Once *Trh* expression is initiated, the protein must heterodimerize with the *Drosophila* *Arnt* (*DArnt*)/Tango (*Tgo*) gene product in order to translocate to the nucleus and activate transcription of nuclear target genes, including itself. *Trh* must initiate an autoregulatory feedback loop in order to amplify the initial signal and then maintain that level of expression for efficient activation of transcription of downstream targets. *Tgo*, also a bHLH-PAS protein, is

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the homologue of the mammalian Arnt gene which functions as a dimerization partner for the aromatic hydrocarbon receptor (Ahr) transcription factor in mammals. Once heterodimerized and inside the nucleus, the Trh::Tgo transcriptional activator recognizes a *cis*-regulatory CNS midline element (CME) in the target genes that are destined for tracheal-specific transcription. Trh is needed for expression of many tracheal-specific genes including the *branchless* (Btl) FGF receptor and the *tracheae defective* (tdf) bZIP transcription factor. Btl binds the *branchless* (btl) ligand and is required for the non-directionally-specific, chemotactic outgrowth of the primary branches towards the sites of highest *btl* expression. The expression of other genes such as *thick veins* and *rhomboid*, which are transcriptionally activated by Vvl, and the expression of their target genes (*dpp* and *spitz*, respectively) acts to determine the directional migratory fate of the primary branches. The *sprouty* gene appears to be activated in response to Btl/Bnl activity and acts to limit branching activity to the region of highest *btl* activation.

#### Example 4

A genetic screen as described above is performed against a collection of P-element induced mutant fly lines as well as other lethals with the intention of identifying genes that interact with *Dakt1*. A mutant fly line, PI193 is determined to result in an average of 7.6% lethality by ectopic PCD while in *trans* to *Dakt1*. This mutant is a P-element insertion in the 5' untranslated region of the *DPP2A* gene at cytological region 61C of chromosome two. Ectopic PCD is associated with the dead embryos from the *DPP2A/Dakt1* cross when assayed for PCD by the standard methods described above.

To determine if *DPP2A* protein is a substrate for PKB/AKT/*Dakt1* kinase activity, a kinase assay is performed as described in Example 2 and in the detailed description of FIG. 5 with PKB as the kinase and *tracheless* protein or fragments thereof as the test substrates.

All citations referred to in the specification are incorporated herein in their entirety as though their relevant contents had been directly reproduced herein.

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## CLAIMS

1. A method of determining whether a mutation capable of enhancing programmed cell death is present in a *Drosophila melanogaster* fly, the method comprising:
  - (a) providing a mutant first fly having a recessive first mutation, the presence of which mutation is known to induce programmed cell death in an embryo of a fly homozygous for the mutation;
  - (b) providing a second mutant fly for determination;
  - (c) crossing the first and second flies;
  - (d) determining whether embryos obtained in step (c) have undergone ectopic programmed cell death;
  - (e) wherein, if the embryos obtained in step (d) have undergone ectopic cell death then a mutation capable of enhancing programmed cell death is present in the second fly.
2. The method of claim 1, wherein the first fly is known to have at least one mutation in a *DAkt1* gene known to induce programmed cell death in an embryo of a fly homozygous for the mutation.
3. The method of claim 2, wherein the at least one mutation in the *DAkt1* gene results in loss of kinase activity of the protein encoded thereby.
4. The method of claim 3, wherein the at least one mutation results in a phenylalanine to isoleucine substitution at position 327 of the said protein.
5. The method of claim 4, wherein the mutation is a TTC→ATC.
6. The method of claim 1, wherein the first fly is known to have at least one mutation in a *DPP2A* gene known to induce programmed cell death.
7. The method of claim 6, wherein the at least one mutation in the *DPP2A* gene results in loss of phosphatase activity of the protein encoded thereby.
8. The method of any preceding claim, wherein the second fly is obtained by exposing a wild-type fly to ionizing radiation, by chemical mutagenesis of a wild-type fly, or by inserting P-elements in the genome of a wild-type fly.
9. The method of any of claims 1 to 8, wherein step (d) includes visually examining embryos obtained in step (c) for cuticle loss.

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10. The method of claim 9, further comprising the step of treating embryos having cuticle loss by degrading or removing the chorion of said embryos to permit staining of embryonic cells interior of the chorion.
11. The method of claim 10, further comprising the step of staining the treated embryos with a dye and determining whether said embryos have undergone programmed cell death by visual inspection of said embryos.
- 5 12. The method of claim 11, further comprising determining whether said treated embryos have undergone programmed cell death by assaying by terminal deoxy-transferase mediated hapten-labeled-dUTP nick end labeling or with acridine orange.
13. The method of any preceding claim, further comprising the step of determining the chromosomal location a mutation in the second fly.
- 10 14. The method of claim 13, further comprising the step of determining the nucleotide sequence of the located mutation.
15. The method of claim 14, further comprising the step of sequencing the chromosome of the fly in the vicinity of said mutation and comparing the sequence obtained with a wild type sequence to determine the wild type sequence corresponding to the mutant sequence.
- 15 16. The method of claim 15, further comprising the step of, if the wild type sequence is a known sequence, determining whether a function of the wild type sequence is known.
17. The method of claim 16, further comprising the step of determining the coding portion of the wild type sequence.
18. The method of claim 17, further comprising the step of comparing the coding portion with known sequences of other species and selecting a sequence which potentially encodes a protein having a function in common with a protein encoded by the fly wild type sequence.
- 20 19. The method of claim 15, further comprising the step of, if the wild type sequence is not a known sequence, comparing the sequence of the mutant fly in the vicinity of said mutation with sequences of other species and selecting a sequence which potentially encodes a protein having a function in common with a protein encoded by the fly wild type sequence.
- 25 20. The method of claim 19, further comprising the steps of creating a nucleic acid molecule for use as a probe and screening a library of nucleic acid molecules with the probe.
21. The method of any preceding claim, further comprising the step of determining the phenotype of the dead embryos after step (e).

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22. The method of any preceding claims, wherein step (c) includes determining whether at least 5 about percent, more preferably at least about 6 percent, more preferably at least about 7 percent, of embryos have undergone ectopic cell death.
23. A method of determining whether a mutation capable of disrupting development is present in a *Drosophila melanogaster* fly, the method comprising:
- (a) providing a first mutant fly having a recessive first mutation, the presence of which mutation is known to induce programmed cell death in an embryo of a fly homozygous for the mutation;
  - (b) providing a second mutant fly for determination;
  - (c) crossing the flies in (a) and (b);
  - 10 (d) examining dead embryos obtained in step (c) to determine whether dead embryos of a phenotype different from either the first or second mutant flies are present;
  - (e) wherein, if a said different phenotype is observed then a mutation capable of disrupting development is present in the second mutant fly.
24. The method of claim 23, wherein the first fly is known to have at least one mutation in a *D4kt1* gene known to induce programmed cell death in an embryo of a fly homozygous for the mutation.
25. The method of claim 24, wherein the at least one mutation in the *D4kt1* gene results in loss of kinase activity of the protein encoded thereby.
26. The method of claim 25, wherein the at least one mutation results in a phenylalanine to isoleucine substitution at position 327 of the said protein.
27. The method of claim 26, wherein the mutation is a TTC→ATC.
28. The method of claim 23, wherein the first fly is known to have at least one mutation in a *DPP2A* gene known to induce programmed cell death.
29. The method of claim 28, wherein the at least one mutation in the *DPP2A* gene results in loss of phosphatase activity of the protein encoded thereby.
30. The method of any of claims 23 to 29, wherein the second fly is obtained by exposing a wild-type fly to ionizing radiation, by chemical mutagenesis of a wild-type fly, or by inserting P-elements in the genome of a wild-type fly.

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31. The method of any of claims 23 to 30, wherein step (d) includes visually examining embryos obtained in step (c) to determine whether trachea are missing from the embryos.
32. The method of any of claims 23 to 31, further comprising the step of determining the chromosomal location a said mutation in the second fly.
- 5 33. The method of claim 32, further comprising the step of determining the nucleotide sequence of the located mutation.
34. The method of claim 33, further comprising the step of sequencing the chromosome of the fly in the vicinity of said mutation and comparing the sequence obtained with a wild type sequence to determine the wild type sequence corresponding to the mutant sequence.
- 10 35. The method of claim 34, further comprising the step of, if the wild type sequence is a known sequence, determining whether a function of the wild type sequence is known.
36. The method of claim 35, further comprising the step of determining the coding portion of the wild type sequence.
37. The method of claim 36, further comprising the step of comparing the coding portion with known  
15 sequences of other species and selecting a sequence which potentially encodes a protein having a function in common with a protein encoded by the fly wild type sequence.
38. The method of claim 34, further comprising the step of, if the wild type sequence is not a known sequence, comparing the sequence of the mutant fly in the vicinity of said mutation with sequences of other species and selecting a sequence which potentially encodes a protein having a function in common with a protein encoded by the  
20 fly wild type sequence.
39. The method of claim 38, further comprising the steps of creating a nucleic acid molecule for use as a probe and screening a library of nucleic acid molecules with the probe.
40. The method of any of claims 23 to 39, further comprising the step of determining the phenotype of the dead embryos after step (e).
- 25 41. The method of any of claims 23 to 40, wherein step (c) includes determining whether at least 3 about percent, more preferably at least about 4 percent, more preferably at least about 5 percent, more preferably at least about 6 percent, of dead embryos have a phenotype in common with each other and different from either the first or second mutant flies.
42. A method of determining whether a mutation capable of disrupting development is present in a *Drosophila*  
30 *melanogaster* fly, the method comprising:

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- (a) providing a first mutant fly having a recessive first mutation, the presence of which mutation is known to disrupt development in an embryo of a fly homozygous for the mutation;
- (b) providing a second mutant fly for determination;
- (c) crossing the flies in (a) and (b);
- 5 (d) examining dead embryos obtained in step (c) to determine whether dead embryos of a phenotype different from either the first or second mutant flies are present;
- (e) wherein, if a said different phenotype is present then a mutation capable of disrupting development is present in the second mutant fly.
43. The method of claim 42, wherein the first fly is known to have at least one mutation in a *trachealess* gene known to disrupt development in an embryo of a fly homozygous for the mutation.
- 10 44. The method of claim 43, wherein the mutation comprises a P-element insertion in the 5' untranslated region of the *trachealess* gene at cytological region 61C of chromosome three.
45. The method of any of claims 42 to 44, wherein the second fly is obtained by exposing a wild-type fly to ionizing radiation, by chemical mutagenesis of a wild-type fly, or by inserting P-elements in the genome of a wild-type fly.
- 15 46. The method of any of claims 42 to 44, wherein step (d) includes visually examining embryos obtained in step (c) to determine whether embryos missing trachea are present.
47. The method of any of claims 23 to 46, further comprising the step of determining the chromosomal location a said mutation in the second fly.
- 20 48. The method of claim 47, further comprising the step of determining the nucleotide sequence of the located mutation.
49. The method of claim 48, further comprising the step of sequencing the chromosome of the fly in the vicinity of said mutation and comparing the sequence obtained with a wild type sequence to determine the wild type sequence corresponding to the mutant sequence.
- 25 50. The method of claim 49, further comprising the step of, if the wild type sequence is a known sequence, determining whether a function of the wild type sequence is known.
51. The method of claim 50, further comprising the step of determining the coding portion of the wild type sequence.



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52. The method of claim 51, further comprising the step of comparing the coding portion with known sequences of other species and selecting a sequence which potentially encodes a protein having a function in common with a protein encoded by the fly wild type sequence.
53. The method of claim 49, further comprising the step of, if the wild type sequence is not a known sequence,  
5 comparing the sequence of the mutant fly in the vicinity of said mutation with sequences of other species and selecting a sequence which potentially encodes a protein having a function in common with a protein encoded by the fly wild type sequence.
54. The method of claim 53, further comprising the steps of creating a nucleic acid molecule for use as a probe and screening a library of nucleic acid molecules with the probe.
- 10 55. The method of any of claims 42 to 54, further comprising the step of determining the phenotype of the dead embryos after step (e).
56. The method of any of claims 42 to 55, wherein step (c) includes determining whether at least 5 about percent, more preferably at least about 6 percent, more preferably at least about 7 percent, of dead embryos have a phenotype in common with each other and different from either the first or second mutant flies.
- 15 57. The method of any of claims 1 to 12, 23 to 32, 42 to 44 or 46, wherein a marker has been introduced into the chromosome of the mutant second fly at a location suitable to permit identification of a mutation introduced into the second fly, and wherein the method further comprises the step of identifying the mutation.
58. The method of claim 57, wherein the step of identifying the mutation includes sequencing the chromosome of the fly in the vicinity of said marker and comparing the sequence obtained with a wild type sequence to determine  
20 the wild type sequence corresponding to the mutant sequence.
59. The method of claim 58, further comprising the step of determining whether a function of the wild type sequence is known.
60. The method of claim 58, further comprising the step of determining the coding portion of the wild type sequence.
- 25 61. The method of claim 60, further comprising the step of comparing the coding portion with known sequences of other species and selecting a sequence which potentially encodes a protein having a function in common with a protein encoded by the fly wild type sequence.
62. A method of inducing programmed cell death in a cell by inhibiting the kinase activity of *Dakt1* protein.
63. The method of claim 62, including administering a nucleic acid molecule sufficiently complementary such  
30 as to inhibit translation of *Dakt1* mRNA present in a cell.

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64. A method of inducing programmed cell death in a cell by inhibiting the phosphatase activity of a *PP2A* protein.
65. The method of claim 64, including administering a nucleic acid molecule sufficiently complementary such as to inhibit translation of *PP2A* mRNA present in a cell.
- 5 66. An assay for determining whether a substance is potentially suitable for usage as an active ingredient in inducing programmed cell death or in inhibiting cell death, which assay comprises the following steps:
- exposing *D4kt* having kinase activity to the substance;
- quantitatively assaying the effect of the substance on the kinase activity; and
- determining based on the results of said assay the potential suitability of the substance as an active  
10 ingredient in inducing programmed cell death or in inhibiting programmed cell death.
67. The assay of claim 66, wherein a said substance is potentially suitable as an active ingredient in inducing programmed cell death if determined to quantitatively inhibit the kinase activity.
68. The assay of claim 66, wherein a said substance is potentially suitable as an active ingredient in inhibiting programmed cell death if determined to quantitatively enhance the kinase activity.
- 15 69. The assay of claim 66, further comprising the following steps:
- exposing embryos of *Drosophila melanogaster* fly to the substance;
- quantitatively assaying the effect of the substance on ectopic cell death in the embryos; and
- determining based on the results of said assay the potential suitability of the substance as an active  
ingredient in inducing programmed cell death or inhibiting programmed cell death.
- 20 70. An assay for determining whether a substance is potentially suitable for usage as an active ingredient in inducing programmed cell death or inhibiting programmed cell death, which assay comprises the following steps:
- exposing *PP2A* having phosphatase activity to the substance;
- quantitatively assaying the effect of the substance on the phosphatase activity; and
- determining based on the results of said assay the potential suitability of the substance as an active  
25 ingredient in inducing programmed cell death or in inhibiting programmed cell death.
71. The assay of claim 70, wherein a said substance is potentially suitable as an active ingredient in inducing programmed cell death if determined to quantitatively inhibit the phosphatase activity.
72. The assay of claim 70, wherein a said substance is potentially suitable as an active ingredient in inhibiting programmed cell death if determined to quantitatively enhance the phosphatase activity.

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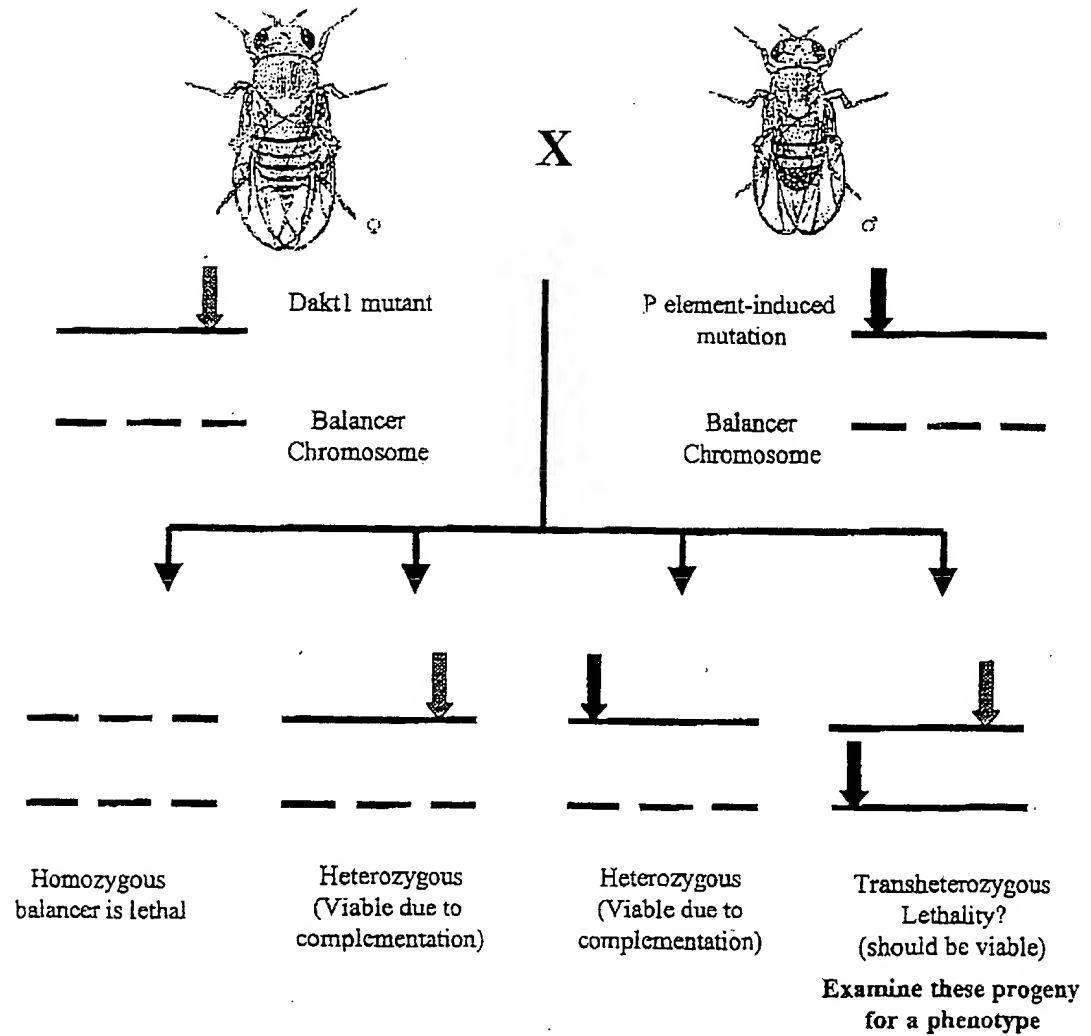
73. The assay of claim 70, further comprising the following steps:  
exposing embryos of *Drosophila melanogaster* fly to the substance;  
quantitatively assaying the effect of the substance on the ectopic cell death in the embryos; and  
determining based on the results of said assay the potential suitability of the substance as an active  
ingredient in inducing programmed cell death or in inhibiting programmed cell death.
74. An assay for determining whether a substance is potentially suitable for usage as an active ingredient in  
arresting development of an organism, which assay comprises the following steps:  
exposing a *trachealess* protein capable of initiating transcription to the substance;  
quantitatively assaying the effect of the substance on the ability of the protein to initiate transcription; and  
determining based on the results of said assay the potential suitability of the substance as an active  
ingredient in arresting development of an organism.
75. The assay of claim 74, further comprising the following steps:  
exposing embryos of *Drosophila melanogaster* fly to the substance;  
quantitatively assaying the effect of the substance on development of the embryos; and  
determining based on the results of said assay the potential suitability of the substance as an active  
ingredient in arresting development of an organism.
76. The assay of claim 75, wherein assaying the effect of the substance on development of the embryos  
includes determining the effect of the substance on tracheal development.
77. A method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for  
abnormal development, the method comprising the steps of:  
providing a sample of genetic material of the subject;  
determining whether the genetic material includes a nucleotide sequence encoding a mutant *trachealess*  
protein incapable of initiating transcription, wherein  
the presence of said nucleotide sequence indicates the presence of a potential to confer on its offspring a  
predisposition for abnormal development.
78. The method of claim 77, further comprising the step of:  
determining whether the genetic material includes a nucleotide sequence encoding a *trachealess* protein  
capable of initiating transcription.
79. The method of claim 77, wherein the *trachealess* protein capable of initiating transcription includes the  
amino sequence identified as SEQ ID NO:1.

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80. A method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for abnormal development, the method comprising the steps of:  
providing a sample of genetic material of the subject;  
determining whether the genetic material includes a nucleotide sequence encoding a mutant PP2A protein  
5 lacking phosphatase activity, wherein  
the presence of said nucleotide sequence indicates said potential predisposition.
81. A method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for abnormal development, the method comprising the steps of:  
providing a sample of genetic material of the subject;  
10 alternatively: determining whether the genetic material includes a nucleotide sequence encoding a mutant  
DAkT/PKB protein having kinase activity lower than the activity of the corresponding wild type  
DAkT/PKB of the subject, wherein]  
the presence of said nucleotide sequence indicates said potential predisposition.
82. A method of inducing programmed cell death in a cell, the method comprising inhibiting the activity of  
15 PP2A in the cell.
83. The method of claim 82, wherein inhibiting the activity of PP2A in the cell includes administering to the cell an effective amount of an antisense nucleic acid molecule sufficiently complementary to an mRNA encoding PP2A to bind thereto so as reduce translation thereof.
84. The method of claim 82, wherein inhibiting the activity of PP2A in the cell includes administering to the  
20 cell an effective amount of a phosphatase inhibitor.
85. The method of claim 84 wherein the inhibitor is selected from the group consisting of microcystin LR and caliculin.
86. An assay for determining whether a substance is potentially suitable for usage as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death, which assay comprises the following steps:  
25 providing a cell in which a reporter gene is operably linked to a promoter/enhancer which is acted upon by  
*trachealess*, wherein the reporter gene is one which, in nature, is not operably linked to the  
sequence promoter/enhancer;  
  
exposing the cell to the substance;  
  
quantitatively assaying the effect of the substance on expression of the reporter gene; and  
  
30 determining based on the results of said assay the potential suitability of the substance as an active  
ingredient in inducing programmed cell death or in inhibiting programmed cell death.

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87. The assay of claim 86, wherein the cell is a cell of a *Drosophila melanogaster* fly.
88. The assay of claim 87, wherein the promoter/enhancer includes the nucleotide sequence SEQ ID NO:3.
89. The assay of any of claims 86 to 88, wherein the determination step includes comparing the effect of the substance on the expression of the reporter gene with the expression of the reporter gene in the absence of the substance.
90. The assay of any of claims 86 to 89, wherein the substance is a kinase inhibitor or a phosphatase inhibitor.
91. The assay of any of claims 86 to 90, wherein a said substance is potentially suitable for usage as an active ingredient in inducing programmed cell death if determined to quantitatively inhibit expression of the reporter gene.
92. The assay of any of claims 86 to 91, wherein a said substance is potentially suitable for usage as an active ingredient in inhibiting programmed cell death if determined to quantitatively enhance expression of the reporter gene.
93. An assay for determining whether a substance is potentially suitable for usage as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death, which assay comprises the following steps:  
exposing a cell to the substance;  
quantitatively assaying the effect of the substance on expression of the *trachealess* gene; and  
determining based on the results of said assay the potential suitability of the substance as an active ingredient in inducing programmed cell death or in inhibiting programmed cell death.
94. The assay of claim 93, wherein the assaying step includes determining the amount of RNA encoding *trachealess* produced by the cell in the presence of the substance and comparing the amount with the amount of RNA encoding *trachealess* produced by the cell in the absence of the substance.
95. The assay of claim 94 wherein determining the amount of RNA encoding *trachealess* includes amplifying the RNA in the presence of primers complementary thereto.
96. The assay of claim 94, wherein a said substance is potentially suitable as an active ingredient in inducing programmed cell death if determined to quantitatively inhibit said expression.
97. The assay of claim 94, wherein a said substance is potentially suitable as an active ingredient in inhibiting programmed cell death if determined to quantitatively enhance said expression.



**Figure:** Schematic representation of the genetic screen performed to identify mutations that interact with *Dakt1*. As shown above, marked balancer chromosomes are used in both fly lines that carry the *Dakt1* and the P-element insertion in order to prevent recombination.

**FIGURE 1**

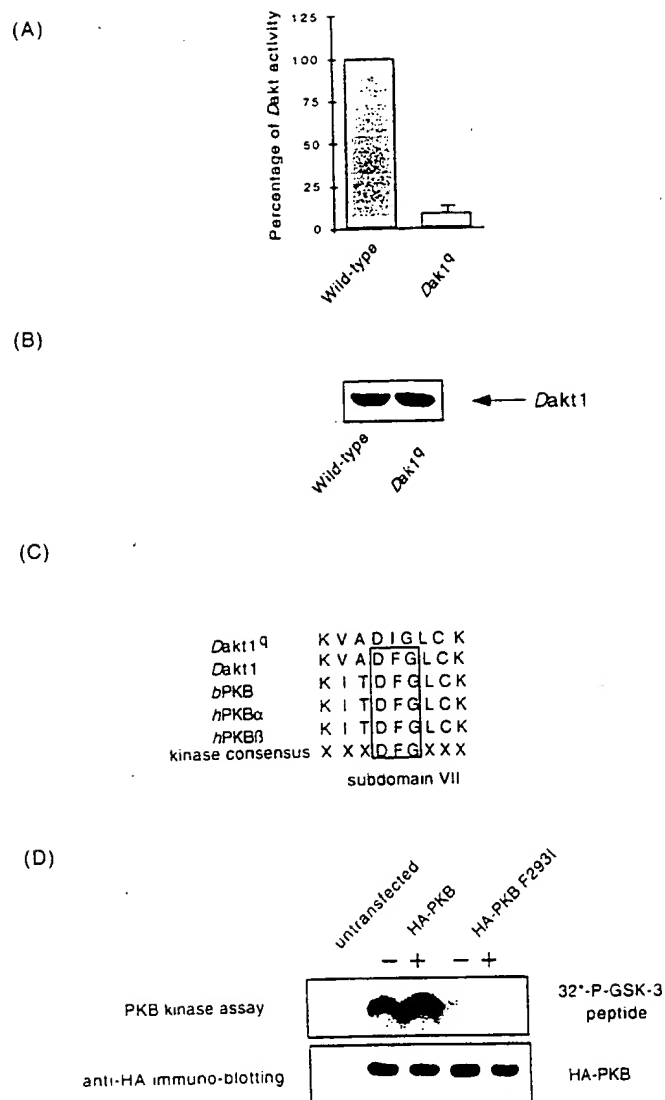


FIGURE 2

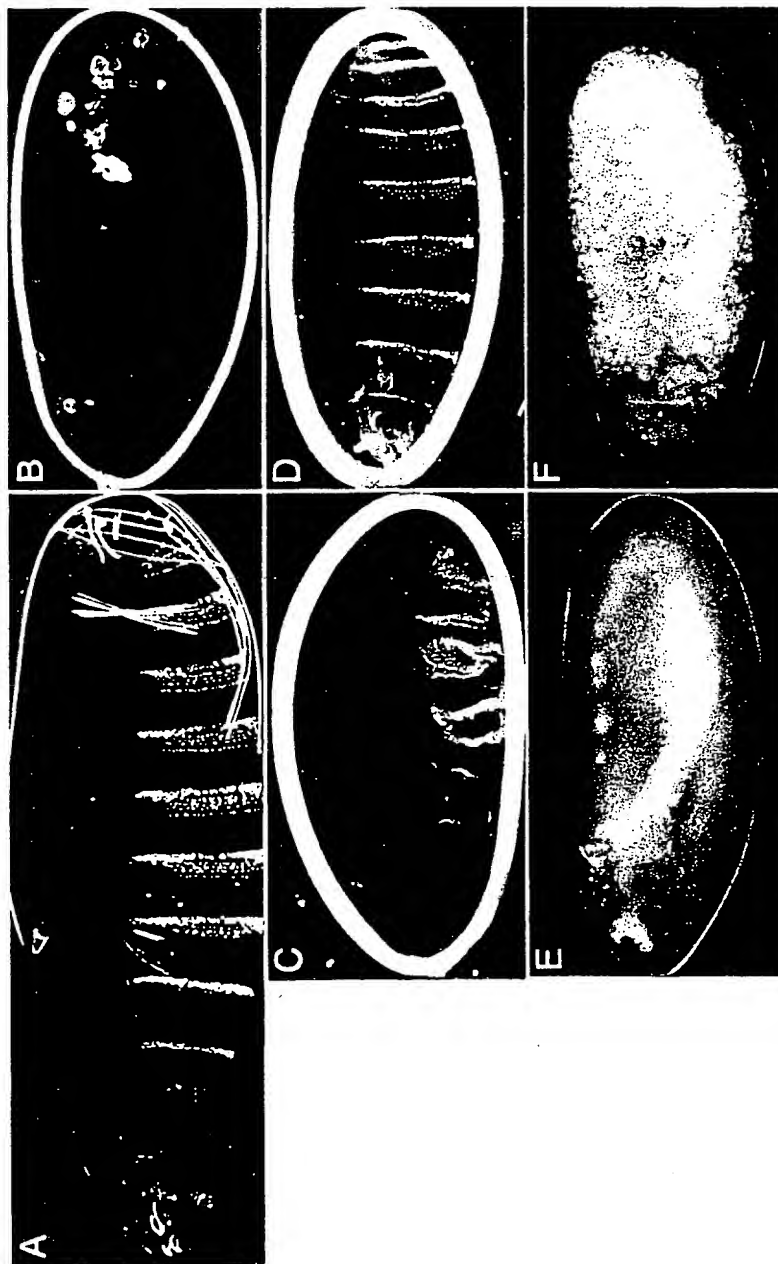


FIGURE 3



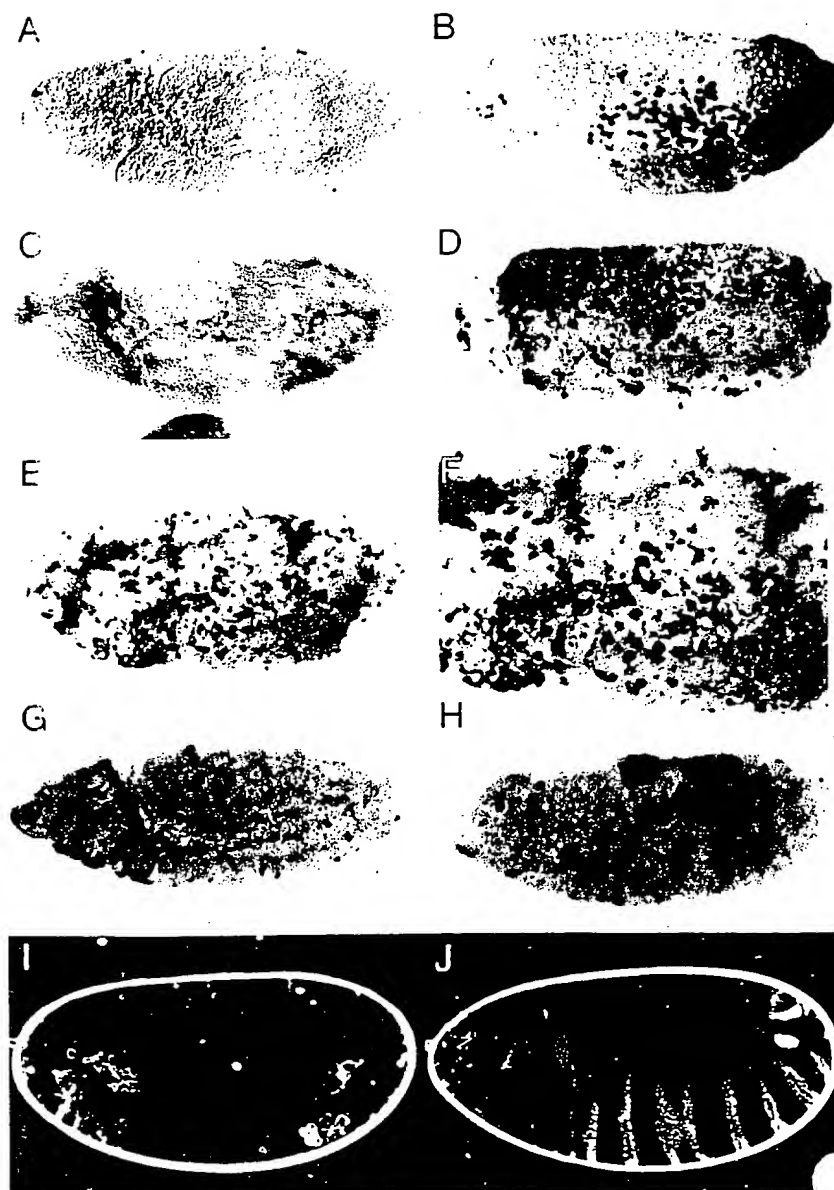


FIGURE 4

Phosphorylation of *trachealess* by PKB (I)

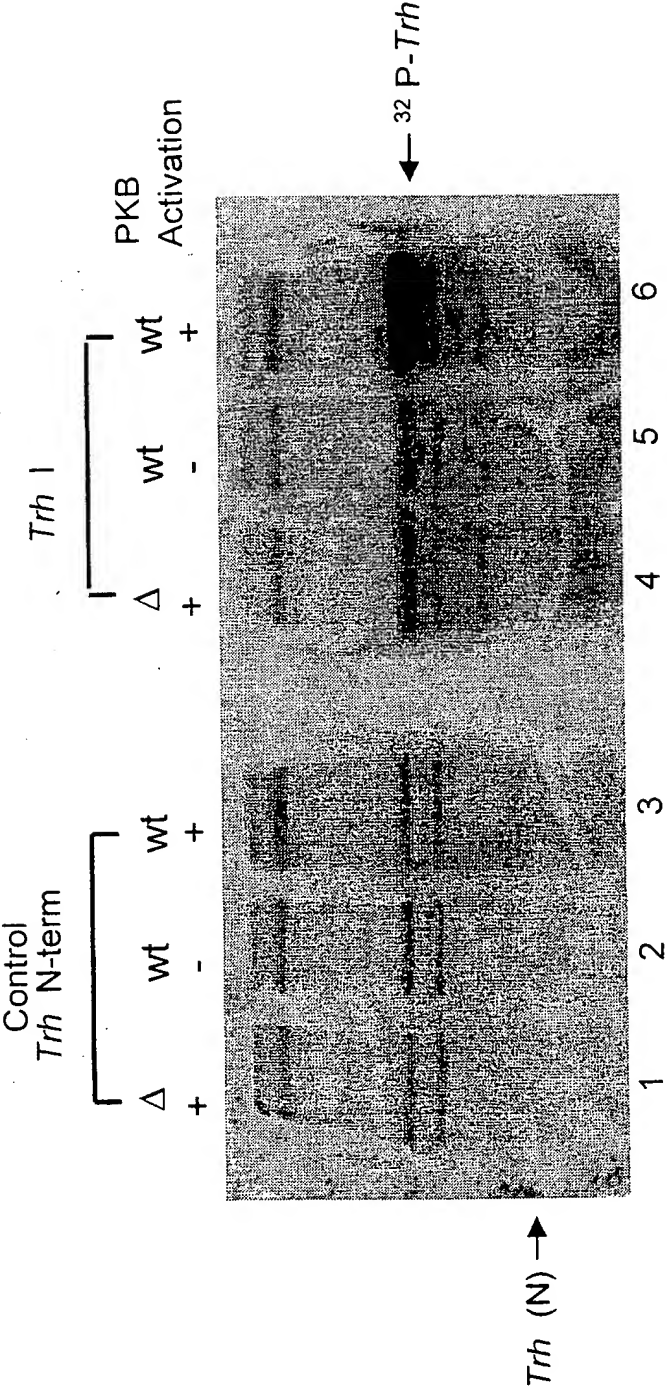


FIGURE 5

Phosphorylation of *trachealess* by PKB (II)

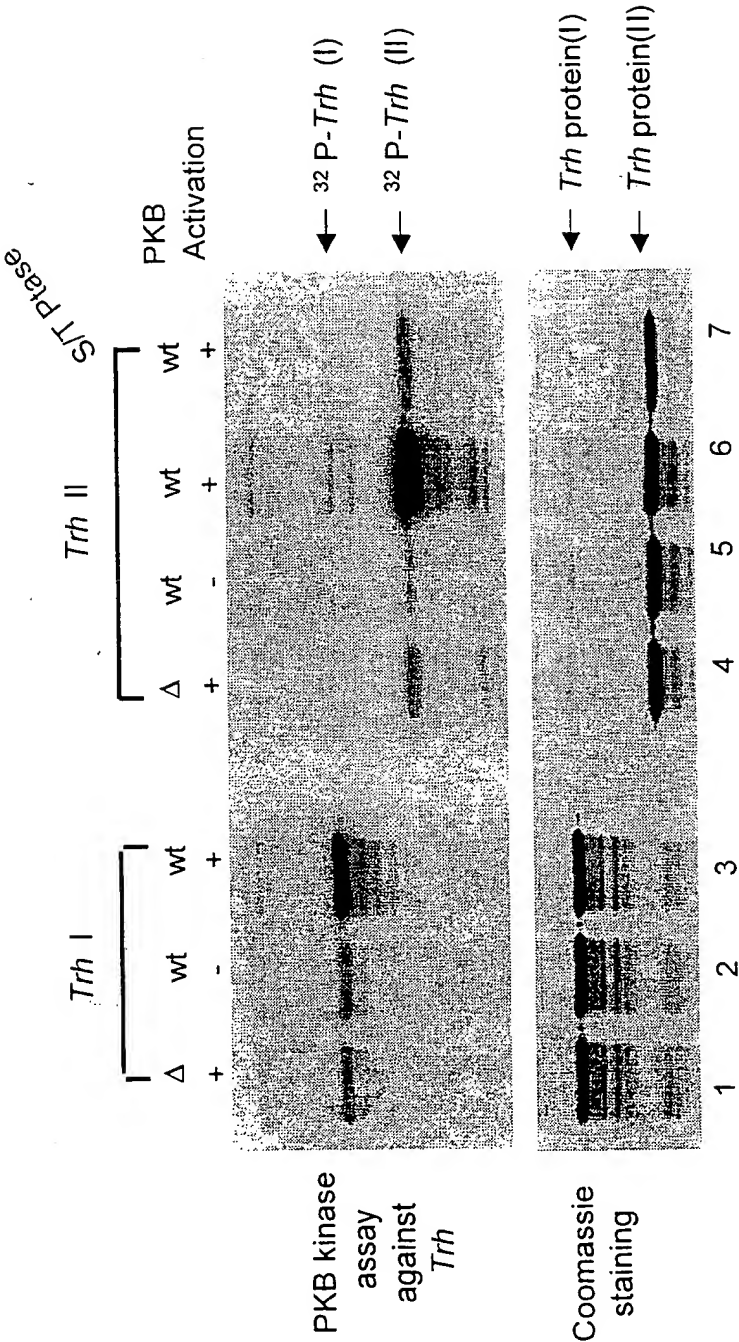


FIGURE 6

PKB specifically phosphorylates *trachealess* at serine665

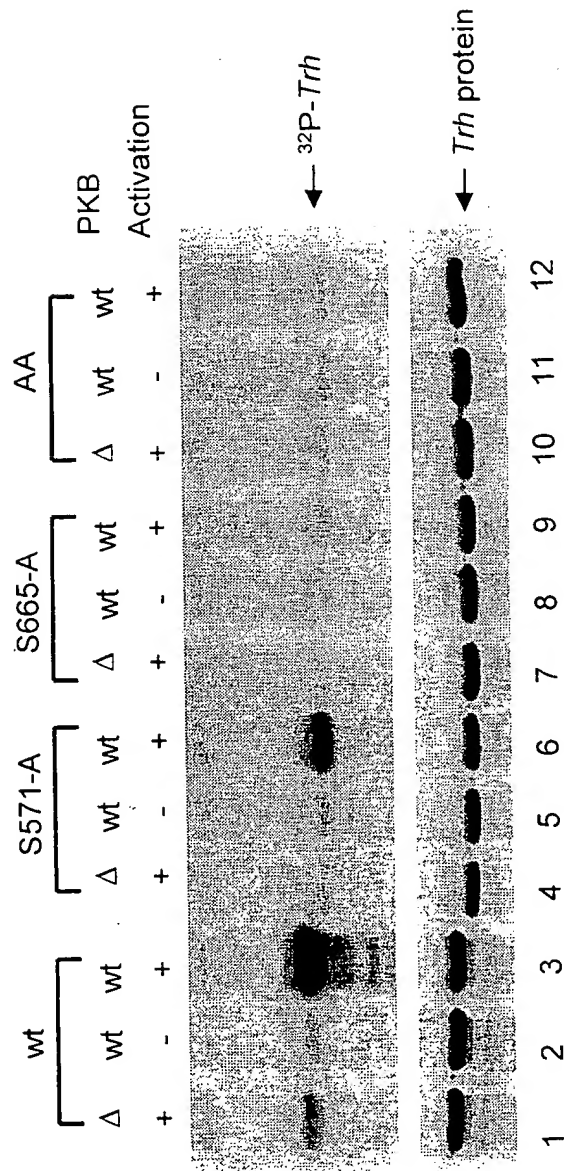


FIGURE 7

*Trachealess* / dARNT (*tango*) hetero-dimer transactivates  
*Drosophila* *breathless* gene, and promotes trachea  
development.

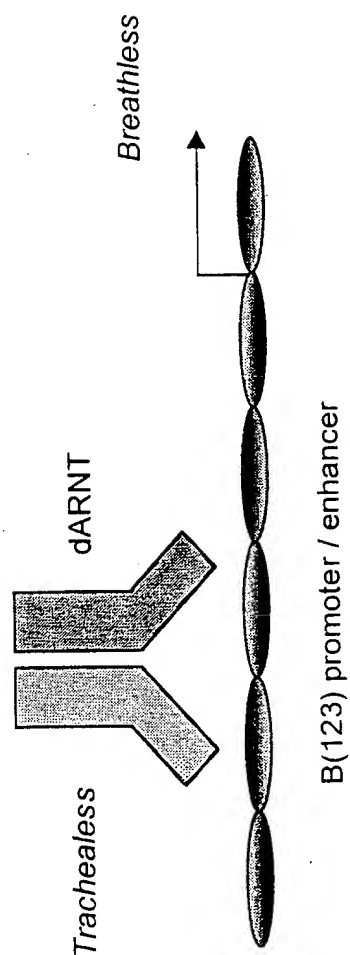


FIGURE 8

The regulation of *tracheless* / dARNT  
transactivation via S665 of *tracheless*

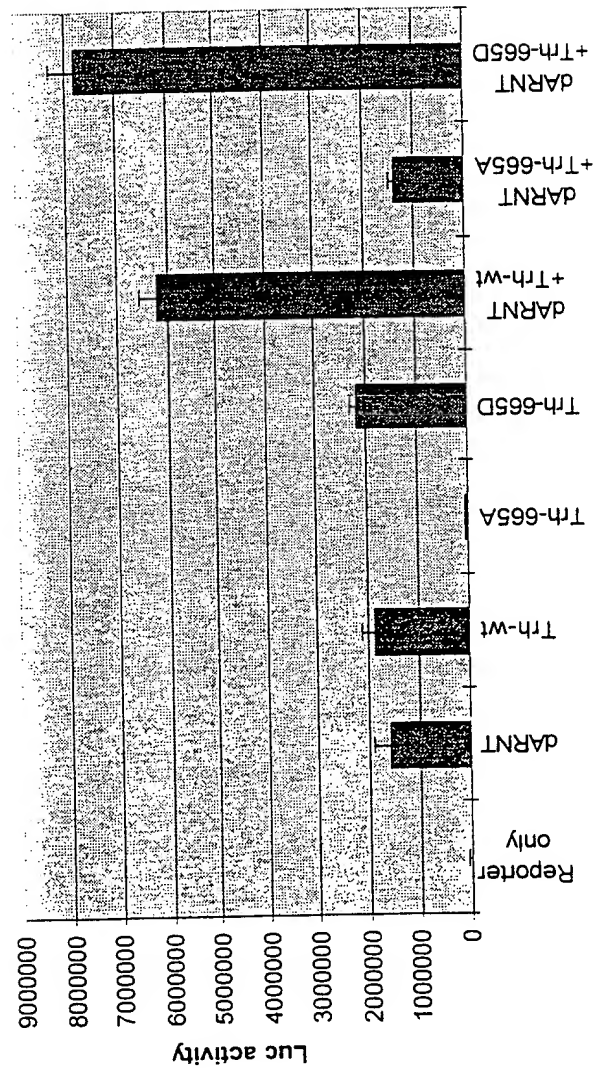


FIGURE 9

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SEQ. ID NO. 1

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121 agttttcacgt acgcagcccc gccgtgcaag tgtgtccgta tatccgtagg ccatctcttt  
181 cgctcacccc ccttttcgaa gggaagtcag tgcgtgtccg taattggcag gtctcattcc  
241 gcagaacgca ggggcacgaa cgtagcgtag ccatagcccc ttaaccgtaa ccagtaaccc  
301 ggagacgtgc caagtgtcgg agataaatta actggacgcg aagcgctcat ttgaatgcca  
361 atcgattgac ggctagtgtg ccgatcccga tccgtattgc gtgaccgta catcgctggc  
421 tggattcgag gataactaaa gacacgtacc acacgtgcgt gtgtgattac gtttatgtga  
481 cgcggaacaa aaaagtatgc ggccttcaaa tcgaatgcca cgcacctcg aatccgtgcc  
541 ccaagccaag tgcaataaca agtgatagag tgccagaaaag tagttgaaaa ctctctgaga  
601 tcacaggct tccaataaga aaaaaggctt agccccagca ttgctatgtt gccgtaccag  
661 gcagccgtgg ccatggacta cgccggatac cagagacagc ccacgcggga catcccgga  
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2101 tgtcgtctgc agcacaaaga acgccgatga gcagaacatc atctgcgtga actatgtgat  
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4381 tattacctct aagagttcag ctaacgtttg tctatttcog tttcctccct attgtatatt  
4441 taatgaaacc ttctaataaa atctagttaa aaatacatca acaacaaaat gccaaaggcaa  
4501 gaaaagctaa aaagtgtaat taaattttgt ataaacgcaa agaaagtttc tcccattaaa  
4561 ggctt

4/5

SEQ. ID NO. 2

"MPAVPFTHSWMVPTQDLCAMPPYNKMTGHQQPPGAGMHAQQQPL  
EPGILELRKEKSRDAARSRRGKENYEFYELAKMLPLPAAITSQLDKASIIRLTISYLK  
LRDFSGHGDPPWTREASSSSKLKSAAIRRSPAVDLFEQHQQTHILQSLDGFALAVAAD  
GRFLYISETVSIYLGLSQVEMTGSSIFDYIHQADHSEIADQLGLSLTSGGGGGGGSS  
SSGGGGGGAGGGMASPTSGASDDGSGTHGTNNPDVAASMTQASTSGYKGYDRSFCVRM  
KSTLTKRCHFKSSGYRASDATSNCCNNGNNAKNVKNPGSNYSVLLCLLRPQY  
TFSHSRKSQPPLGLMVALAIALPPPSVHEIRLECDMFVTRVNFDLRVAHCEPRVSDLL  
DYSPELDVNKSLYSLCHAEDANRLRKSHSDLIEKGQVLTGYRMLNKS GGYTWLQTCA  
TVVCSTKNADEQNIICVNYVISNRENENMILDCCQLESPDSIKHEEGLGNDKSSGSP  
GGDASGEGNSHLSAGDMKLNSPKTDSEGHSHRGRGRSAAASHGSSMNSLTMIKDSPTP  
LGVEIDSGVLEPTTVATPVPAATPPVQSTKRKRKTKASQHAEDQGQEQVISEQPLPKLP  
TMEQRDQQPRSRLPSTIVDEQPSSAADS AVKDLEQAMSKHLPSPAAVSVVAPPNTDFSA  
DSLLKQQQQQQQLDPNEKSSTIQWIGTPYQQPPAPMPATALLRQLYANRESVIRATAR  
QTPTGVGPGVIFYGDQQTGPLTPPGSESSYENQYLQLHSAASGGHPGGQKTSADAFTN  
LVSTYGGYHSSIDYHNAMTPSSVSPRDSNQPGKAAPVLASNGGYDYAPDPLRGQYAT  
SSGDVVPATLPLKPQASYTATMHPSGSTTTTEGGVTYSNLDQPQYFAPHSSFHL YHKGS  
PASGWYSTPS"

5/5

SEQ. ID NO. 3

5' -gagaaagagatgttcaaatttaagaactaatatcttttcgcattctgacctcggtaaacatgggtgttggt  
aagaaggcaccgaccaaatagaagacatcaggtgagtttggttattaattcacggaaattgtgttagctatacc  
aagttcttgctagctagtttgatagtaagttccctccttttcgtatttcccaactgttcgcatttgttccca  
tagcccctaacaagtgcataaaaaatgcatttaactatttttgggtagattacgtgcccggcatctggatcagg  
at tt tggcctatgcgggagctagctgctgccatgccacatagcagcaactgagattttggatatcagtcacgt  
aacggatatttccttattgatatgcatgattaccgcgcagtcacacgtactgcacaccactaagacaatggacacat  
tttcttttgcataaattaagccgccaagcaaaacacagggagggaaacccggccagataataaagaatttcaac  
gagtcgaagccaccgcaaactgcttagaataaaaaccaacaagaacctagcaggagcagcaacagcagcaaac  
agcaattaaaaaagaaaggaaaccgctcaggaaaactaccaagcgaaaacaattgcaacatgcgggagggagac  
cacctcgatcaggtagccgtccgaacccataccatcccaaccgaagacctgtgcccaggatcctagcaaacatt  
atcgcagggaaaccacagatacccaacgatacccaactcgttttcgtatcggtatcggtatcggttcggttcgaa  
cggcacgttcggatcggttcgtatcggtaccgcgtaaaagtttcttcggttcggtatcggttactttcagttgttgc  
gcacattcaagccaatcggttggttcggtcgattccttcacaaaactttacggattacgggttagtgcaacgaa  
gtgaccccccccccccc-3'

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12Q 1/68</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 99/55906</b> <b>(43) International Publication Date:</b> 4 November 1999 (04.11.99)
<b>(21) International Application Number:</b> PCT/CA99/00377 <b>(22) International Filing Date:</b> 26 April 1999 (26.04.99) <b>(30) Priority Data:</b> 60/083,077 27 April 1998 (27.04.98) US <b>(71) Applicant (for all designated States except US):</b> THE TORONTO HOSPITAL [CA/CA]; 610 University Avenue, Toronto, Ontario M5G 2M9 (CA). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> WOODGETT, James, R. [GB/CA]; 19 Lynedock Crescent, Don Mills, Ontario M3A 2A7 (CA). MANOUKIAN, Armen, S. [CA/CA]; 59 Paperbirch Drive, North York, Ontario M3C 2E6 (CA). JING, Jin [CN/CA]; Apartment 905, 30 Charles Street West, Toronto, Ontario M4Y 1R5 (CA). WETSCH, Ben [CA/CA]; 593 Huron Street, Toronto, Ontario M5R 2R8 (CA). <b>(74) Agent:</b> HUNT, John, C.; Blake, Cassels & Graydon, Commerce Court West, P.O. Box 25, Toronto, Ontario M5L 1A9 (CA).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 16 March 2000 (16.03.00)
<b>(54) Title:</b> GENE SCREENING METHODS AND RELATED ASSAYS  <b>(57) Abstract</b>  The present invention relates to cell death genes, which are genes required for programmed cell death; mutant organisms, in which ectopic programmed cell death occurs in embryos to a greater than normal extent and agents which alter the ability to modulate programmed cell death of cells. A mutant form of <i>Dakt1</i> , <i>Dakt1<sup>u</sup></i> in a <i>Drosophila melanogaster</i> has been identified. <i>Dakt1</i> and its mammalian homolog, PKB are known to protect cells from undergoing apoptosis. A genetic screen for identifying genes related to programmed cell death by their ability to interact genetically with the mutant form of <i>Dakt1</i> is described.		

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# INTERNATIONAL SEARCH REPORT

Int. onal Application No

PCT/CA 99/00377

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 40173 A (CHIRON CORP)	64,65,
Y	30 October 1997 (1997-10-30)	68-71,83
	the whole document	2-7,
		9-23,
		25-30,
		32-44,
		47-63,
		67,73,
		75,82,85
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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

12 January 2000

Date of mailing of the international search report

28. 01. 2000

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Hagenmaier, S

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00377

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HEITZLER ET AL.: "A GENETIC ANALYSIS OF PANNIER, A GENE NECESSARY FOR VIABILITY OF DORSAL TISSUES AND BRISTLE POSITIONING IN DROSOPHILA" GENETICS, vol. 143, 1996, pages 1271-1286, XP002115023	1,24
Y	the whole document	2-5, 9-23, 25-28, 32-43, 48-63, 68-71,83
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X	LAWRENCE: "THE MAKING OF A FLY" 1992, BLACKWELL SCIENCE, LONDON XP002115027 page 131 -page 132	1,24
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# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/CA 99/00377

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00377

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A	MCCALL K ET AL: "Facing death in the fly: genetic analysis of apoptosis in Drosophila" TRENDS IN GENETICS, vol. 13, no. 6, 1 June 1997 (1997-06-01), page 222-226 XP004065317 ISSN: 0168-9525 the whole document ---	
A	WO 94 16071 A (MASSACHUSETTS INST TECHNOLOGY) 21 July 1994 (1994-07-21) the whole document ---	
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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/00377

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 06116 A (UNIV MINNESOTA) 29 February 1996 (1996-02-29) the whole document ---	
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P,X	STAVELEY ET AL.: "GENETIC ANALYSIS OF PROTEIN KINASE B (AKT) IN DROSOPHILA" CURRENT BIOLOGY, vol. 8, no. 10, 27 April 1998 (1998-04-27), pages 599-602, XP002115025 the whole document ---	2-5, 9-23, 25-28, 32-44, 47-63, 68-71,83
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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/CA 99/00377

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
  
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
  
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/CA 99/00377

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 1-5, 9, 24-28, 44, 64, 65, 68-71,  
83 (all completely) and 10-23, 32-43,  
47-63 (all partially)

Use of a mutation in the DAkt1 gene in a first fly in a method of determining whether a mutation capable of enhancing programmed cell death or disrupting development is present in a second fly of *Drosophila melanogaster*, a method of inducing programmed cell death in a cell by inhibiting kinase activity of DAkt1, use of DAkt1 protein to determine whether a substance is potentially suitable for usage as an active ingredient in inducing or inhibiting programmed cell death by exposing Dakt having kinase activity to the substance and quantitatively assaying the effect of the substance on the kinase activity, and a method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for abnormal development providing a sample of genetic material of the subject, and determining whether the genetic material includes a nucleotide sequence encoding a mutant form of DAkt1.

2. Claims: 6, 7, 29, 30, 66, 67, 72-75, 82,  
84-87 (all completely) and 10-23, 32-43,  
49-63 (all partially)

Use of a mutation in the DPP2A gene in a first fly in a method of determining whether a mutation capable of enhancing programmed cell death or disrupting development is present in a second fly of *Drosophila melanogaster*, a method of inducing programmed cell death in a cell by inhibiting phosphatase activity of PP2A, use of PP2A protein to determine whether a substance is potentially suitable for usage as an active ingredient in inducing or inhibiting programmed cell death by exposing PP2A having phosphatase activity to the substance and quantitatively assaying the effect of the substance on the phosphatase activity, and a method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for abnormal development providing a sample of genetic material of the subject and determining whether the genetic material includes a nucleotide sequence encoding a mutant form of PP2A.

3. Claims: 45, 46, 76-81,  
88-100 (all completely) and 47-63 (all partially)

Use of a mutation in the trachealess gene in a first fly in

## INTERNATIONAL SEARCH REPORT

International Application No. PCT/CA 99/00377

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

a method of determining whether a mutation capable of disrupting development is present in a second fly of *Drosophila melanogaster*, use of trachealess to determine whether a substance is potentially suitable for usage as an active ingredient in inducing or inhibiting programmed cell death by assaying the effects of a substance on the transcription initiating activity of trachealess or the expression of the trachealess gene and a method of predicting in a subject the presence of a potential to confer on its offspring a predisposition for abnormal development providing a sample of genetic material of the subject and determining whether the genetic material includes a nucleotide sequence encoding a mutant form of trachealess.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 99/00377

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